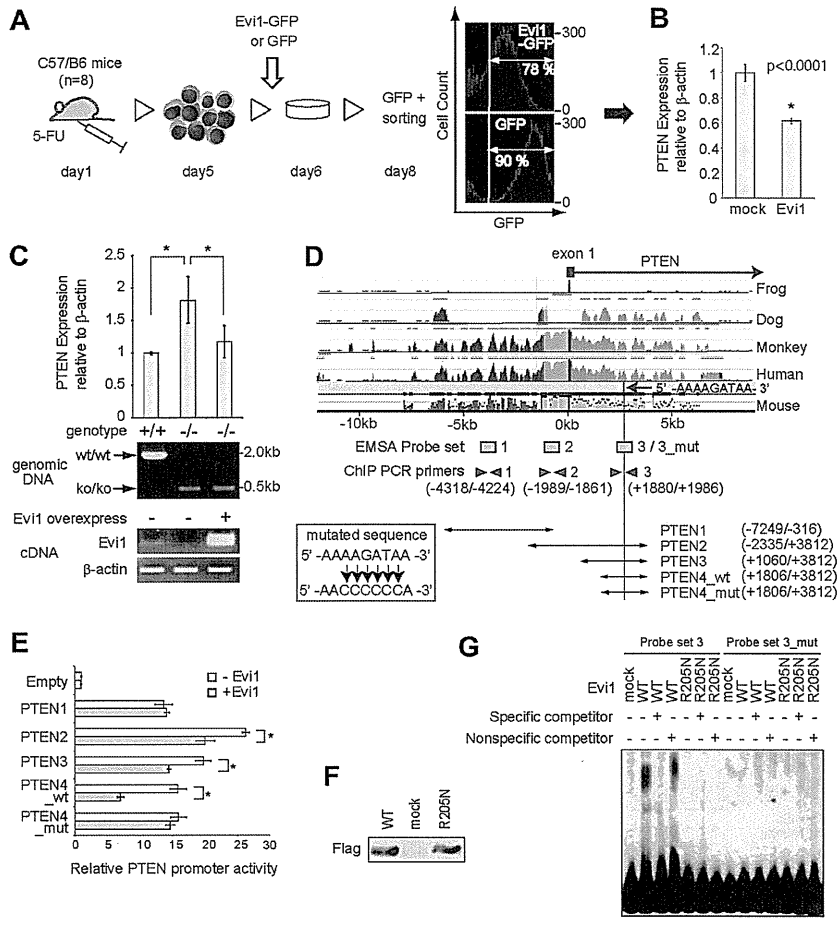

Figure 1. Evi1 down-regulates PTEN expression in BM cells. (A) Schematic representation of gene expression analysis. Evi1-GFP– or GFP-transduced BM cells (n = 4 for each) were sorted and subjected to gene expression analysis. Representative fluorescence-activated cell sorting data show the gene transfer efficiencies of 78% and 90% for Evi1-GFP and GFP-expressing retrovirus, respectively. (B) Real-time PCR for PTEN expression. Error bars indicate SD (n = 6; $*P < .0001$). (C) PTEN mRNA expression in lineage$^-$, c-Kit$^+$, Sca-1$^+$ cells derived from Mx-Cre *Evi1*$^{flox/flox}$ mice. Error bars indicate SD (n = 3; $*P < .05$). Evi1 was retrovirally added back. The Cre-mediated *Evi1* deletion and the expression of wild-type Evi1 were evaluated by PCR with the use of genome DNA and cDNA, respectively, and representative figures are shown. Detailed experimental methods are shown in supplemental Data. (D) Schematic representation of mouse PTEN promoter region, possible Evi1 binding site predicted by rVISTA 2.0 (http://rvista.dcode.org/), constructs cloned into pGL4.10[Luc2], mutagenesis strategy, probe sets used for EMSAs, and primers for ChIP assays. (E) Relative luciferase activity of Evi1 on each PTEN promoter. Error bars indicate SD (n = 6; $*P < .01$). Jurkat cells were used. Error bars indicate SD. (F) Protein expression of Flag-tagged wild-type (WT) Evi1 and Evi1_R205N used for EMSAs. Western blot analysis was done with anti-Flag antibody. (G) Results of EMSAs are shown. Probe sets 3 or 3_mut are indicated in Figure 1D. Corresponding cold-specific competitors or nonspecific competitors were added as indicated. KO indicates knockout.

wild-type C57/B6 mice were transduced with Evi1-GFP or GFP-expressing retroviruses, and GFP$^+$ cells were sorted and subjected to gene expression profiling (Figure 1A). To narrow down the candidate genes, we assessed the correlation between expression of Evi1 and the potential target genes in AML samples with the use of published gene expression data of 285 persons with AML.[6] When we looked at the genes that showed both ≥ 1.3-fold decrease in the expression value of Evi1-GFP–transduced cells ($P < .05$; n = 8) and the inverse correlation with Evi1 in human AML ($P < .01$; n = 285) (see supplemental Methods for detailed information), we noted that 3 probe sets for PTEN appeared in the list (supplemental Table 1). PTEN transcription was inversely correlated with that of Evi1 in 285 AML samples (supplemental Table 2). A similar trend was observed when we analyzed another published dataset of 43 patients with AML with lower statistical power[38] (supplemental Table 2). We confirmed the above data by quantitative real-time PCR analysis (Figure 1B). We also found that PTEN expression was higher in *Evi1*-deficient lineage$^-$, c-Kit$^+$, Sca-1$^+$ cells than in *Evi1*$^{+/+}$ lineage$^-$, c-Kit$^+$, Sca-1$^+$ cells, which was recovered by forced expression of Evi1 (Figure 1C).

To determine whether Evi1 regulates the transcription of PTEN, we next performed luciferase reporter assays. The genomic region of ~ 10-kb base pairs around the transcription start site (TSS) of PTEN is well conserved across human and mouse, and 2 putative binding sequences for the first zinc finger (ZF1-7) domain of Evi1 were predicted by rVISTA 2.0 with the use of a matrix similarity threshold of 0.80 (http://rvista.dcode.org/). The one is located at position −4257/−4243 and has the [GAC/TA] N_{0-6} [GAT/CA]–like motif[2] (matrix similarity = 0.80), and the other one is located at position 1935/1943 and contains the GACAAGATA-like motif[39] (matrix similarity = 0.85). Thus, we divided this region of murine PTEN promoter into 2 fragments (PTEN1 and PTEN2; Figure 1D) and inserted them into pGL4.10 [luc2]. Each reporter plasmid was transiently transfected into Jurkat cells, in which endogenous Evi1 expression is low (data not shown), with or without Evi1 expression plasmid. Evi1 achieved an ~ 0.25-fold decrease of luciferase activity over basal levels with the PTEN2 constructs (Figure 1E). Stepwise deletion constructs of PTEN reporter (PTEN3 and PTEN4_wt) showed that Evi1 represses PTEN transcription through the region between 1.8 and 3.8 kb downstream of the TSS. Then we created a promoter construct carrying mutated Evi1-binding sites (PTEN4_mut) placed at position 1935/1943, which harbors the evolutionarily conserved GACAAGATA-like sequence (Figure 1D). As shown in Figure 1E, the mutated promoter was insensitive to Evi1 expression, indicating that this sequence is responsible for the effect of Evi1 on PTEN regulation. We obtained essentially the same results in other cell lines, THP-1 and COS7 (supplemental Figure 1A-B).

We next performed EMSAs to test whether Evi1 binds to the GACAAGATA-like motif located at position 1935/1943 downstream of the PTEN TSS. Murine Evi1_R205N harbors a single amino acid change within the ZF1-7 domain and was previously reported to be incapable of binding to DNA containing the GACAAGATA-like motif.[39] Flag-tagged wild-type Evi1 and Evi1_R205N were transfected in 293T cells, purified with immunoprecipitation with the use of anti-Flag affinity gel (Figure 1F), and

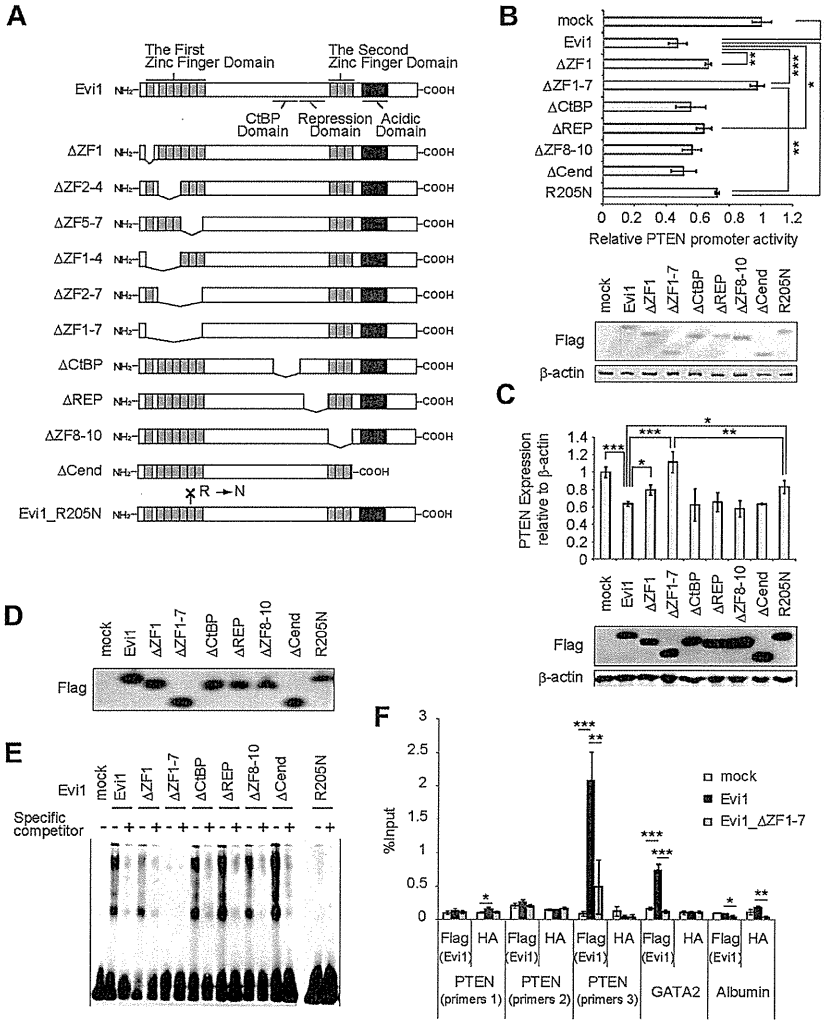

Figure 2. Evi1 represses PTEN expression via its first zinc finger domain. (A) Schematic representation of Evi1 and its mutants. (B) Relative luciferase activity of Evi1 and its mutants on PTEN4_wt promoter (n = 4) together with immunoblot of assayed Jurkat cells transfected with Flag-tagged wild-type Evi1, its mutants, and mock. Error bars indicate SD; $*P < .05$, $**P < .01$, and $***P < .001$. (C) PTEN mRNA expression in Flag-tagged Evi1-, its mutants-, or mock-transduced BM cells together with protein expression of Evi1 and its mutants (n = 3). Error bars indicate SD; $*P < .05$, $**P < .01$, and $***P < .001$. (D) Protein expression of Flag-tagged wild-type Evi1 and its mutants used in EMSAs. Western blot analysis was done with anti-Flag antibody. (E) EMSAs used mutants of human Evi1 and the probe set 3. (F) ChIP analysis for Flag-Evi1, Flag-Evi1_ΔZF1-7, or mock-expressing BM cells that used the indicated antibodies (n = 3). Error bars indicate SD; $*P < .05$, $**P < .01$, and $***P < .001$. REP, repression domain; Cend, domain at the C-terminus.

applied to EMSA. Wild-type Evi1 formed a specific DNA-protein complex with the probe set 3 that contains murine PTEN promoter sequence with the AAAAGATAA motif, and it was weakened by cold-specific competitors but not by nonspecific competitors (Figure 1G). Wild-type Evi1 did not bind to the probe set 1 or 2 that was set upstream of the probe set 3 (supplemental Figure 2). However, Evi1_R205N failed to bind to the probe set 3, and wild-type Evi1 did not bind to the probe set 3_mut, whose sequence was mutated in the same way as PTEN4_mut that was used in the reporter assays. These results indicate that Evi1 specifically interacts with the GACAAGATA-like motif within the PTEN promoter via its ZF1-7 domain.

Next, we analyzed transcriptional activities of a series of Evi1 mutants[40] (Figure 2A). The ZF1-7 domain is a DNA-binding domain and is essential for interaction with several proteins, including Sma and Mad related protein 3 (*SMAD3*)[8] and c-Jun N-terminal kinase.[9] The second zinc finger (ZF8-10) domain is another DNA-binding domain and is essential for activator protein-1 activation.[10] The repression domain is required for the efficient repression of transforming growth factor-β signaling.[8] The region containing CtBP-binding motif-like sequences is responsible for the interaction with CtBP1.[19] In addition, Evi1 contains a highly acidic domain at the C-terminus, which is required for Evi1-mediated P-Sp hematopoiesis.[17] The deletion of ZF1-7 almost completely abolishes the repressive activity of Evi1 in Jurkat cells, (Figure 2B), THP-1 cells (supplemental Figure 1C), and COS7 cells (supplemental Figure 1D). Evi1_ΔZF1 and Evi1_R205N have partially lost repressive effects on the PTEN promoter. The murine homologue of these mutants (Evi1_ΔZF1, Evi1_ΔZF1-7, or Evi1_R205N) did not fully repress PTEN transcription when it was retrovirally transduced in primary BM cells (Figure 2C). Moreover, EMSAs suggested that Evi1_ΔZF1-7 did not bind to the PTEN promoter (the probe set 3) (Figure 2D-E). Thus, ZF1-7 domain of Evi1 plays a major role for PTEN repression, although there is a possibility that the other domains of Evi1 and/or the other sites within ZF1-7 make additional contribution.

We further tested whether Evi1 binds to the PTEN promoter in primary BM cells. ChIP assays that used Flag-Evi1, Flag-Evi1_ΔZF1-7, or mock-transduced BM cells showed that Evi1 was significantly enriched in the region containing Evi1-binding sequence, which was amplified with primers 3 (Figure 1D) but not in the other regions of the PTEN promoter (amplified with primers 1 or 2) or in the irrelevant albumin promoter (Figure 2F). The association of Evi1 with the PTEN promoter was stronger than that detected with GATA2 promoter, a well-established target of Evi1.[2] In contrast, we observed little to no enrichment of Evi1_ΔZF1-7 in the promoter of PTEN or GATA2. No enrichment was detected with the use of an unrelated antibody (anti-HA). Taken together,

these data suggest that Evi1 binds to the PTEN promoter through ZF1-7 domain to repress its transcription.

Evi1 activates the AKT/mTOR pathway

We then asked whether Evi1 represses protein expression of PTEN and induces the activation of the downstream AKT/mTOR signaling pathway in primary BM cells. We prepared Evi1- or mock-transduced BM cells and investigated the status of this pathway. As shown in Figure 3A, Evi1 decreased the protein level of PTEN and increased the phosphorylation of AKT as well as mTOR. In contrast, Evi1 had little to no effect on the phosphorylation of extracellular signal-regulated kinase 1/2, signal transducer and activator of transcription 3, or signal transducer and activator of transcription 5 (Figure 3B), suggesting that Evi1 selectively activates the PTEN/AKT/mTOR pathway. Next, we compared the status of PTEN/AKT/mTOR signaling in several murine BM cells that were transformed by various oncogenes. Evi1 is known to enhance self-renewal potential,[11] and we could replate Evi1-transduced BM cells > 15 times (data not shown). AML1/ETO, E2A/HLF, and PML/RARα are chimeric genes generated in t(8;21), t(17;19), and t(15;17) leukemias, respectively, all of which are known to transform murine BM cells. With the use of Evi1-, AML1/ETO-, E2A/HLF-, and PML/RARα-, or mock-transduced BM cells, we assessed the status of the PTEN/AKT/mTOR pathway. As shown in Figure 3C and 3D, Evi1-transduced BM cells showed decreased PTEN expression at both mRNA and protein levels and increased phosphorylation of AKT/mTOR compared with AML1/ETO-, E2A/HLF-, PML/RARα-, or mock-transduced cells, indicating that the PTEN/AKT/mTOR pathway is activated in Evi1-transduced BM cells.

We next assessed the effect of rapamycin on colony-forming activity of these oncogene- or mock-transduced BM cells. Evi1-transduced BM cells showed increased sensitivity to rapamycin (half maximal inhibitory concentration < 0.2nM) compared with control cells (half maximal inhibitory concentration > 0.6nM) (Figure 3E). Rapamycin also slightly reduced the colony-forming activity of AML1/ETO-, E2A/HLF-, or PML/RARα-transduced cells, probably through a nonspecific cytotoxic effect. We also treated these cells with phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and obtained similar results (supplemental Figure 3A). We performed similar experiments with inhibitors of the nuclear factor-κB pathway (BMS345541) and the Notch pathway (DAPT), but neither of them has significant effects on Evi1-expressing cells compared with AML1/ETO-, E2A/HLF-, or PML/RARα-transduced BM cells (supplemental Figure 3B-C). We also found that Evi1 overexpression promoted cell cycling progression compared with control cells, and the effect was cancelled by adding the mTOR inhibitor rapamycin (Figure 3F) or overexpression of PTEN (supplemental Figure 4).

To evaluate the effect of rapamycin on Evi1-induced leukemia in vivo, we generated Evi1-expressing leukemic mice by BM transplantation. Wild-type BM cells were transduced with the pMYs-Evi1-IRES-GFP (n = 8; ID: 1-8 shown in supplemental Table 3) or an empty vector (n = 11; ID: 9-19), and were injected into sublethally irradiated mice. All of the recipient mice received a transplant with Evi1-expressing cells died with AML within 6-11 months after transplantation, whereas development of leukemia was not observed in control mice. AMLs were characterized by large numbers of blasts in the BM smear (Figure 3G; supplemental Table 3), positivity for myeloid markers of leukemic cells (Figure 3H), and marked splenomegaly (supplemental Table 3). Because some of the clones were positive for B220, we checked IgH gene rearrangements of leukemic cells, and no clonal J_H rearrangements were detected in both $B220^+$ and $B220^-$ leukemic cells (supplemental Figure 5A). Expression of Evi1 protein was confirmed in these leukemic cells (supplemental Figure 5B). Then isolated leukemic cells (3 clones) were transplanted into sublethally irradiated secondary recipient mice. These mice were treated with daily injections of vehicle (n = 10) or rapamycin (0.4 mg/kg per day; n = 10). Although all of the secondary recipient mice developed AML, rapamycin significantly prolonged the survival of recipient mice compared with vehicle-treated mice (Figure 3I). We also developed murine AML models with the use of TEL/PDGFβR-AML1/ETO and AML1 mutant (AML1_S291fsX300)[35,41] (supplemental Figure 5B-C), but rapamycin did not show any effects on the survival of these mice (Figure 3I). These data suggest that the AKT/mTOR pathway has a role in the proliferation and survival of Evi1-expressing leukemic cells both in vitro and in vivo.

PTEN inversely correlates with Evi1 in human leukemia

To address the potential relevance of the findings to human disease, we analyzed gene expression data of leukemic BM cells of 57 cases with human AML. All samples contained 80%-99% blast cells (Table 1 for the patient characteristics). We evaluated Evi1 and PTEN levels by real-time PCR and found an inverse correlation between Evi1 and PTEN levels with statistical significance (Figure 4A). We further examined Evi1 and PTEN expression in BM cells of CML because high Evi1 expression is observed in patients with CML[4] (n = 44; Table 2). We found that Evi1 seemed to be activated in CML during a blastic phase (Figure 4B), and we identified a statistically significant inverse relationship between Evi1 and PTEN expression levels again, suggesting that PTEN down-regulation by Evi1 may play a role in the progression of the disease from the chronic to the acute phase.

These expression analyses indicate that the inverse correlation between Evi1 and PTEN observed in murine models is recapitulated in human AML and CML.

Evi1 interacts with PcG proteins to repress PTEN

It has been shown that Evi1 recruits several histone methyltransferases (HMTs) for regulation of gene transcription, such as SUV39H1 and G9a.[20-22] EZH2 is another HMT which is a core component of PRC2/3/4 and imparts methyltransferase activity to the complexes. We therefore investigated whether HMTs are actively involved in Evi1-mediated PTEN repression. By the reporter assays that used Jurkat cells, we evaluated the effect of Evi1 on PTEN4_wt promoter in the presence of SUV39H1, G9a, EZH2, and their catalytically inactive mutants. Although SUV39H1 and G9a showed little to no effect on the PTEN repression (Figure 5A-B), Evi1 and EZH2 repressed PTEN promoter activity in a synergistic manner (Figure 5C). Moreover, EZH2-H689A, a construct carrying an inactivating point mutation within the HMT domain of EZH2, completely abolished the transcriptional repression mediated by Evi1. Similar results were obtained with THP-1 and COS7 cells (supplemental Figure 1E and F, respectively). These data suggest that Evi1 requires EZH2 for PTEN down-regulation.

To assess the genetic requirement of EZH2 for Evi1-mediated PTEN regulation in BM cells, we designed 4 independent shRNAs targeting murine EZH2 (shEZH2-A, -B, -C, and -D) and transduced them into Evi1-expressing BM cells. shEZH2-A, -B, and -C strongly reduced EZH2 expression compared with control shRNA, whereas shEZH2-D could not (Figure 5E). PTEN expression was

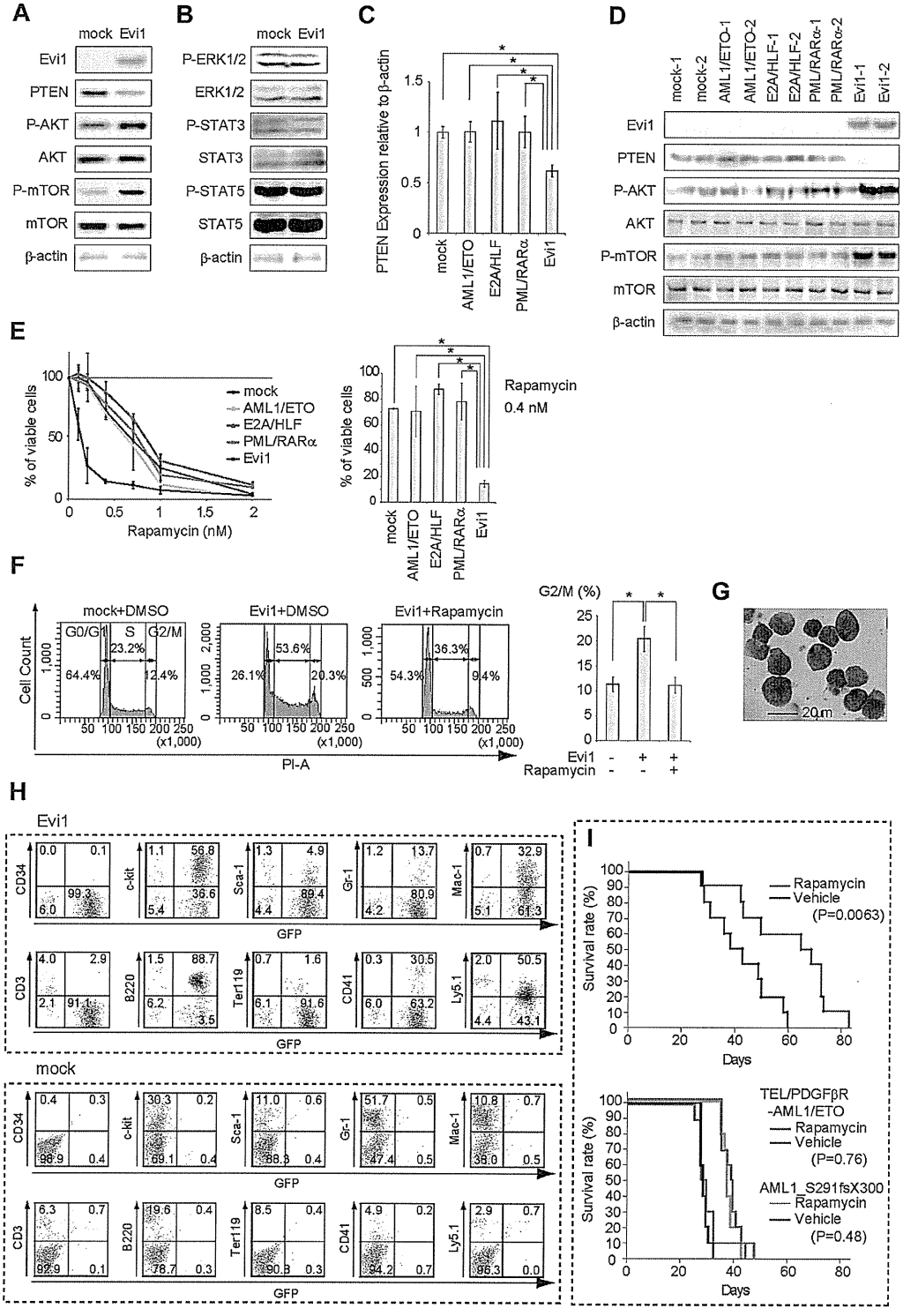

Figure 3. Evi1 represses PTEN protein level and activates downstream AKT/mTOR signaling. (A-B) Analysis of indicated protein levels in Evi1 or mock-transduced BM cells. BM cells ($1\text{-}2 \times 10^6$) were used in these assays. Experiments were repeated for > 2 times, and the representative figures are presented. (C) Comparison of PTEN mRNA expression between various oncogene-transduced BM cells. Mock, AML1/ETO, E2A/HLF, PML/RARα, or Evi1-transduced BM cells were prepared and were analyzed after 1 week of G418 selection. Error bars indicate SD (n = 4; $^*P < .01$). Results were represented as the averages of 4 independent experiments performed in duplicate. (D) Comparison of PTEN/AKT/mTOR signaling of the indicated cells analyzed by Western blotting. BM cells ($1\text{-}2 \times 10^6$) were used in these assays, and 4 independent experiments were performed and representative figures are shown (n = 2 for each). (E) Rapamycin was added to each oncogene- or mock-transduced BM cells with the indicated concentrations in semisolid medium. The average colony counts were converted to percentages, defining the colony number without rapamycin as the cell viability of 100%. *P* values were calculated by comparing percentages of viable cells at 0.4nM rapamycin. Error bars indicate SD (n = 8 from 4 independent experiments; $^*P < .00001$). (F) Cell cycle analysis of Evi1- or mock-transduced BM cells with/without addition of rapamycin. Representative fluorescence-activated cell sorting data (left), and average percentage of cells in G_2/M phase (right) were shown. Error bars indicate SD (n = 3; $^*P < .01$). (G) A BM smear of Evi1-induced AML, stained with Wright-Giemsa, showed an

Table 1. Clinical and molecular characteristics of the 57 patients with AML

Characteristics	Value
Sex, n (%)	
Male	37 (65)
Female	20 (35)
Age group, y, n (%)	
< 35	8 (14)
35-60	22 (32)
> 60	25 (54)
Age, y	
Median	57
Range	16-85
Bone marrow blasts count, %	
Median	87.1
Range	80-99
Disease status, n (%)	
Diagnosis	40 (70)
Relapse 1	8 (14)
Relapse > 2 or refractory	9 (16)
French-American-British classification, n (%)	
M0	4 (7)
M1	12 (21)
M2	19 (39)
M3	4 (7)
M4	5 (9)
M5	7 (12)
M6	1 (4)
M7	0 (0)
Not determined	5 (9)
Cytogenetic abnormalities, n (%)	
t(8;21)	8 (14)
t(15;17)	3 (5)
inv(16)/t(16;16)	2 (4)
t(11q23)	3 (5)
Complex karyotype (> 3 chromosomal abnormalities)	3 (5)
Other abnormal karyotypes*	2 (4)
Normal karyotype	23 (40)
Not determined/no data	13 (23)

*Patients with 3q26 abnormalities are not included in this study.

significantly higher in EZH2-knockdown cells than in control cells at both mRNA and protein levels (Figure 5D-E). Furthermore, EZH2 knockdown resulted in decreased AKT/mTOR phosphorylation (Figure 5E). Thus, EZH2 is genetically required for PTEN down-regulation and subsequent AKT/mTOR activation by Evi1. In contrast, PTEN expression was not changed when these shRNAs were transduced into whole mononuclear BM cells, in which Evi1 expression is low (supplemental Figure 6A-B). Furthermore, overexpression of EZH2 in fluorouracil-primed BM cells did not repress Evi1 mRNA and protein levels (supplemental Figure 6C-D). These results support the idea that EZH2 or polycomb complex is recruited to the PTEN promoter and epigenetically induces repressive chromatin modifications only in cells with high Evi1 expression.

We then performed ChIP assays with the use of murine BM cells transduced with Flag-Evi1, Flag-Evi1_ΔZF1-7, or mock. Evi1 and EZH2 are both enriched in the PTEN promoter only in Evi1-expressing cells (Figure 5F). In addition, strong enrichment of SUZ12, another component of PRC2/3/4, was observed in the same genomic region. Consistent with the binding of PRC2/3/4, H3K27me3 was significantly enriched. Interestingly, BMI1, a component of PRC1, was also detected. Furthermore, we found a decrease in trimethylation of histone H3 lysine 4 and H3 acetylation marks, indicating the repressive epigenetic modification in this genomic region in Evi1-expressing cells. In contrast, we observed no epigenetic modification in other genomic regions, or when we used an unrelated antibody (anti-HA) (supplemental Figure 7A-D). In Evi1_ΔZF1-7–expressing cells, we observed no enrichment of EZH2, SUZ12, BMI1, or H3K27me3 in the PTEN promoter, indicating that ZF1-7 is a central domain for Evi1-mediated PcG recruitment.

To further address the clinical relevance of PcG proteins to PTEN down-regulation, we performed ChIP assays with additionally available samples derived from patients with AML (patients 1-5 indicated in Figure 4A). Primers for human PTEN promoter were designed to amplify fragments that correspond to the murine PTEN promoter regions depicted in Figure 1D. In leukemic cells with high Evi1 expression (patients 4 and 5), Evi1, EZH2, SUZ12, BMI1, and H3K27me3 tended to be enriched in the PTEN promoter (Figure 5G). However, H3K4me3 and H3 acetylation marks had a tendency to be reduced in these cells. Anti-Flag antibody was used as a negative control. These results indicate that Evi1 recruits both PRC2/3/4 and PRC1 to the PTEN promoter region and induces histone modification to repress PTEN transcription in leukemic cells with highly expressed Evi1.

These data prompted us to test whether Evi1 physically interacts with PcG proteins. We introduced HA-tagged Evi1 and components of polycomb complex (Flag-tagged EZH2, Myc-tagged SUZ12, or Flag-tagged EED) into 293T cells. Immunoblot analysis of anti–HA-Evi1 immunoprecipitates showed the existence of Flag-EZH2, Myc-SUZ12, and Flag-EED in a complex with Evi1 protein, under stringent washing condition (500mM NaCl) (Figure 6A, B, and C, respectively). Identical results were obtained by the reciprocal coimmunoprecipitation experiments (supplemental Figure 8A-C). To investigate the existence of the interactions between Evi1 and PcG proteins in leukemic cells, we performed coimmunoprecipitation assays with the use of leukemic cells derived from Evi1-induced leukemia mice and found that Evi1 interacts with PRC2/3/4 proteins in these leukemic cells (Figure 6D). Importantly, similar results were obtained with the use of human leukemia cells derived from the patient with AML with high Evi1 expression (Figure 6E). We next assessed the interaction between Evi1 and PRC1 complexes and found that Evi1 interacts with BMI1, RING1, RING2, and HPH2 (Figure 6F-I; supplemental Figure 9A-D), but immunoglobulin does not interact with HPH1 or HPC proteins (CBX2, CBX4, CBX6, CBX7, and CBX8) (data not shown). In addition, domain-mapping experiments showed that

Figure 3. (continued) increase of myeloblasts. Slides were examined by Olympus BH-2 microscope with 40×/0.75 NA oil objective. Picture was taken with Olympus DP20-E camera and analyzed with Adobe Photoshop 7.0. (H) Representative flow cytometric profiles of the BM cells isolated from a recipient of Evi1- or mock-transduced BM cells. The surface marker profiles of Evi1-induced leukemic cells were almost the same. These cells expressed c-kit and Mac-1. Lymphoid markers such as CD3 and B220 were negative except for some deviations in the intensity of B220. In contrast, mock-transduced cells were hardly detected, which suggested that the transplanted cells did not engraft. The numbers in the figure show the percentage of cells gated in each quadrant. (I) Survival of Evi1-induced leukemic mice (top; n = 20 in total, 3 clones were transplanted), or that of TEL/PDGFβR-AML1/ETO-induced leukemic mice and AML1_S291fsX300-induced leukemic mice (bottom; n = 20 in total for each leukemia, 2 clones were transplanted for each) treated with vehicle or rapamycin. DMSO indicates dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; STAT3, signal transducer and activator of transcription 3; STAT5, signal transducer and activator of transcription 5.

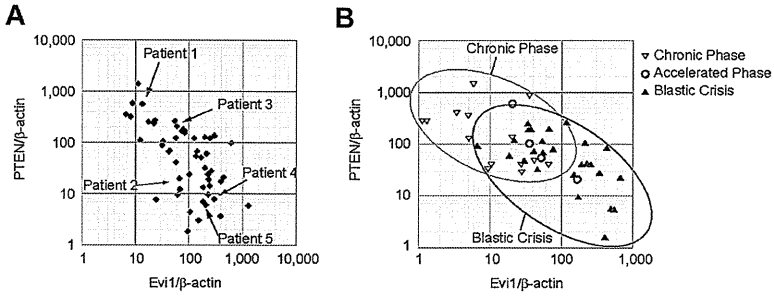

Figure 4. PTEN expression is inversely correlated with Evi1 expression in human leukemia. (A) Correlation between Evi1 and PTEN mRNA expressions in AML (n = 57; Pearson coefficient = −0.339 [P = .0097], Spearman coefficient = −0.298 [P = .024]). The same samples of patients 1-5 were subjected to ChIP analysis (Figure 5G). (B) Correlation between Evi1 and PTEN mRNA expressions in CML with disease status of each patient are shown (n = 44; Pearson coefficient = −0.347 [P = .020], Spearman coefficient = −0.368 [P = .013]).

ZF1-7 of Evi1 is responsible for the interaction with EZH2, EED, and BMI1 (Figure 6J; supplemental Figure 10A and B, respectively), although the interaction between Evi1 and SUZ12 is mediated through multiple regions (supplemental Figure 10C). By more detailed domain-mapping experiments, we found that Evi1 binds to EZH2 via its ZF1 domain (supplemental Figure 10D). Evi1_ΔZF1 can bind to DNA but fails to interact with EZH2, whereas Evi1_R205N can interact with EZH2 but does not bind to DNA (supplemental Figure 10D). These results suggest that complex formation of Evi1 and EZH2 is primarily mediated by protein-protein interaction, rather than by DNA, although we cannot exclude potential participation of DNA. Considering that neither of these mutants efficiently represses PTEN transcription, both binding to DNA and interaction with EZH2 are required for Evi1 to repress PTEN expression.

We further examined whether Evi1 and PcG proteins could colocalize in cells with the use of immunofluorescence analysis, and we found that Evi1 and each PcG protein formed speckles that partially overlap (yellow) in nuclei, confirming the association between the 2 proteins in vivo (supplemental Figure 11A-G).

Myeloid-transforming activity of Evi1 depends on PcG proteins

Finally, we evaluated a role for polycomb complexes in Evi-1–induced myeloid transformation. Evi1- or E2A/HLF-mediated transformed BM cells from the third to fourth round of in vitro replating were infected with retrovirus encoding shRNAs and replated in each dish. Efficient knockdown of EZH2 mediated by shEZH2-A, -B, and -C in Evi1–transduced BM cells significantly reduced their colony-forming activity (Figure 7A). In contrast, knockdown of EZH2 did not impair BM transformation by E2A/HLF. We used E2A/HLF as a control because the contribution of Evi1 to colony-forming activity is relatively small in E2A/HLF-transformed cells.[1] We also designed 4 independent shRNAs for murine SUZ12 and EED, respectively. As shown in Figure 7B, shSUZ12-B, -C, -D and shEED-B, -C, -D strongly reduced the corresponding proteins, whereas other shRNAs showed little effect. Again, we found a significant decrease in colony-forming activity of Evi1-expressing BM cells when we used effective shRNAs for SUZ12 or EED (Figure 7C). We also confirmed that down-regulation of SUZ12 or EED did not affect the colony formation of E2A/HLF-transduced cells. Thus, major components of PRC2/3/4 are specifically required for maintenance of transformation mediated by Evi1.

Table 2. Clinical and molecular characteristics of the 44 patients with CML

Characteristics	Value
Sex, n (%)	
Male	34 (77)
Female	10 (23)
Age group, y, n (%)	
< 35	9 (21)
35-60	27 (61)
> 60	8 (18)
Age, y	
Median	47
Range	17-78
Bone marrow blasts count, %	
Median	3.6
Range	0.5-96
Disease status, n (%)	
Chronic phase	14 (32)
Accelerated phase	4 (9)
Blastic crisis	26 (59)

Patients with 3q26 abnormalities are not included in this study.

Discussion

It is well known that many of the critical regulators of leukemic transformation are transcription factors. Among them is Evi1, and intense attention has been focused on the molecular mechanisms underlying Evi1-mediated transcriptional regulation. The present study showed several important aspects of Evi1-related leukemia.

First, the dependency of Evi1-expressing leukemic cells on AKT/mTOR signaling provides a potential therapeutic target in a genetically distinctive subset of poor-prognosis leukemia defined by high Evi1 expression. Our study clearly showed that inhibition of the AKT/mTOR pathway antagonizes the leukemogenic properties of Evi1-expressing leukemic cells in vitro and in vivo. Thus, rapamycin or other inhibitors of the PI3K/AKT/mTOR signaling will have a therapeutic effect on Evi1-related leukemia. Importantly, it is the first example of targeted therapeutic modalities that suppress the leukemogenic activity of Evi1. Evi1 has a variety of oncogenic potentials, which is probably one of the reasons that rapamycin did not cure the diseased mice in our experiments. However, this will be the first step to overcome Evi1-related leukemia, and the possibility of combination therapy that targets several functions of Evi1 should be tested in the future.

Second, our results strongly suggest that activated Evi1 induces epigenetic regulation on PTEN transcription. Although several studies have shown that the PI3K/AKT pathway is often deregulated in AML,[42] underlying mechanisms of such deregulation are unclear. Some RAS mutations, PTEN mutations, or PTEN phosphorylation can result in AKT activation, but the importance of PTEN in causing AKT activation in AML has remained undetermined.[42] Because histone modifiers or, in particular, PcG proteins that epigenetically regulate PTEN transcription have not been reported in hematologic malignancies, our model will shed light on a new mechanisms of AKT activation in a genetically defined AML

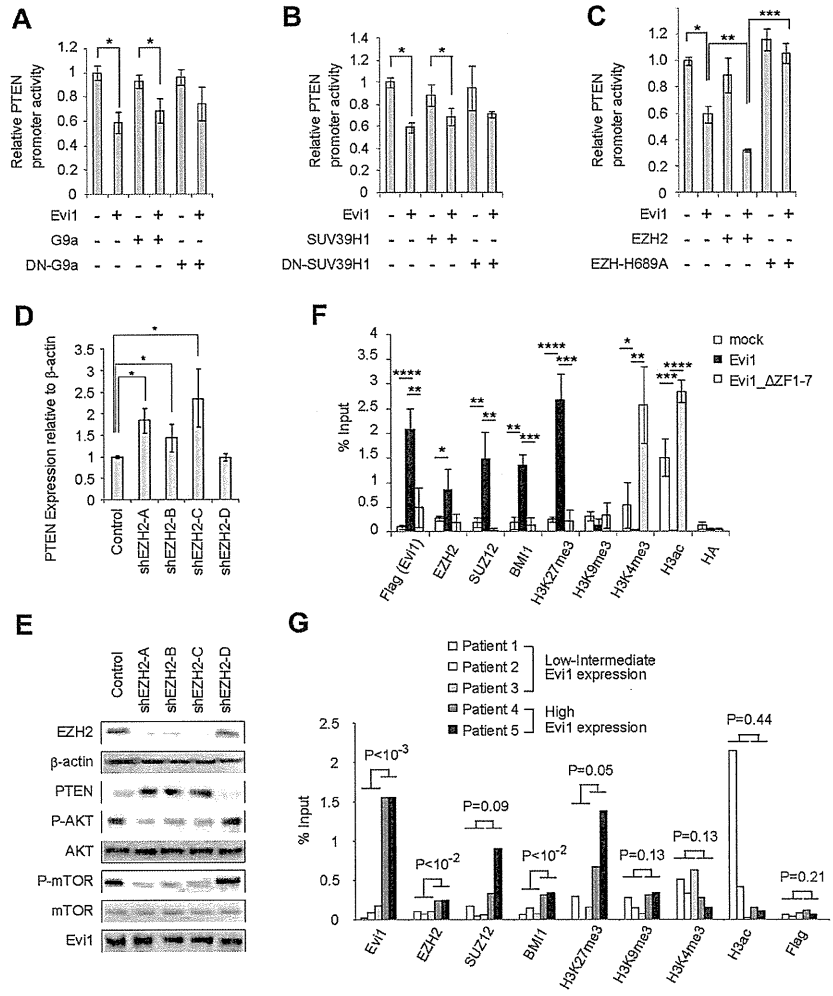

Figure 5. Evi1 recruits polycomb complexes to repress PTEN. (A) Reporter assays that used Jurkat cells and PTEN4_wt promoter in the presence of Evi1, G9a, or dominant-negative (DN) G9a (n = 4). Error bars indicate SD; $*P < .05$. (B) Reporter assays that used Jurkat cells and PTEN4_wt promoter in the presence of EVi1, SUV39H1, or DN-SUV39H1 (n = 4). Error bars indicate SD; $*P < .05$. (C) Reporter assays that used Jurkat cells and PTEN4_wt promoter in the presence of Evi1, EZH2, or EZH2-H689 (n = 6). Error bars indicate SD; $*P < .05$, $**P < .01$, and $***P < .001$. (D-E) shRNAs (shEZH2-A, -B, -C, -D or control) were retrovirally delivered to Evi1-transduced BM cells, and the efficacy of these constructs and the effects on the PTEN/AKT/mTOR signaling were examined by quantitative real-time PCR (D; n = 3) and immunoblotting (E; experiments were performed twice and the representative figures are presented). BM cells ($1\text{-}2 \times 10^6$) were used for Western blotting. Error bars indicate SD; $*P < .05$. (F) ChIP assays for PTEN promoter region as shown in Figure 2F using primers 3 (Figure 1D) (n = 3). Error bars indicate SD; $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$. (G) ChIP assays for PTEN promoter region that used human AML blasts (n = 5). Primers used in these assays amplify sequences, including a putative Evi1 binding site (5′-AGAAGATAA-3′ fragment at position 1875/1884 downstream of the initiation codon ATG [+1] of human PTEN). *P* value was calculated by comparing variables of patients with low-intermediate Evi1 expression (n = 3) and patients with high Evi1 expression (n = 2).

subgroup, which provides a molecular basis for specific treatment (Figure 7D).

Third, note that Evi1 interacts with both PRC2/3/4 and PRC1. The ability of Evi1 to interact with various PcG proteins suggests that Evi1 acts as an anchor for polycomb complexes to DNA to organize gene transcription (Figure 7D). Epigenetic changes mediated by PRC2/3/4 make attractive therapeutic targets because they are potentially reversible processes. In addition, Evi1 also interacts with PRC1 and seems to recruit PRC1 to the PTEN promoter. Recently, Boukarabila et al[43] reported that PLZF/RARα fusion protein interacts with both PRC2/3/4 and PRC1, and the recruitment of PRC1 leads to a deep influence on the maintenance of target gene repression. Therefore, PTEN repression by Evi1 may be firmly maintained by the recruitment of PRC1, and the interactions between Evi1 and PRC1 will be another therapeutic target.

Fourth, PTEN is the first described repressive target of Evi1. Because Evi1 rarely changes transcription levels of genes by ≥ 1.4-fold as shown in our previous gene expression analysis that used Evi1 conditional knockout mice,[1] it is essential to detect subtle changes of gene expression. Several studies have shown the crucial function of PTEN in multiple cellular processes and its involvement in human diseases, which suggests that PTEN needs to be deliberately regulated, and subtle changes in PTEN expression levels have profound effects on tumorigenesis.[44-48] Therefore, we performed an extensive promoter analysis of PTEN. Furthermore, primary BM cells seem appropriate for detecting Evi1 targets in the hematopoietic system because transcriptional regulation by Evi1 is highly context dependent. In fact, we did not find the effect of Evi1 on PTEN in other cell lines, such as 32D, HEK293T, or NIH3T3 cells (data not shown). Recently, Song et al[49] have reported that BMI1 induces epithelial-mesenchymal transition partially through transcriptional repression of PTEN in nasopharyngeal epithelial cells, although it seems yet to be determined whether PTEN is a universal target for BMI1.[49] Our observation in hematopoietic system clearly indicates that BMI1 and other PcG proteins are recruited to the PTEN promoter only in cells with high expression of Evi1 but not in cells with low Evi1 expression. These results show a critical role for Evi1 in anchoring PcG proteins to the PTEN promoter (Figure 7D). Meanwhile, no anchor protein like Evi1 has been identified in BMI1-mediated PTEN regulation in nasopharyngeal epithelial cells or other cells. Because the activated PI3K/PTEN/AKT pathway is well documented for many types of human malignancies and is also associated with an aggressive phenotype,[50] further investigations are warranted for elucidating the epigenetic regulation of PTEN/AKT signaling in various types of cancer cells.

Fifth, we established a novel murine model of Evi1-induced leukemia, which will be a valuable tool for analyzing a mechanistic

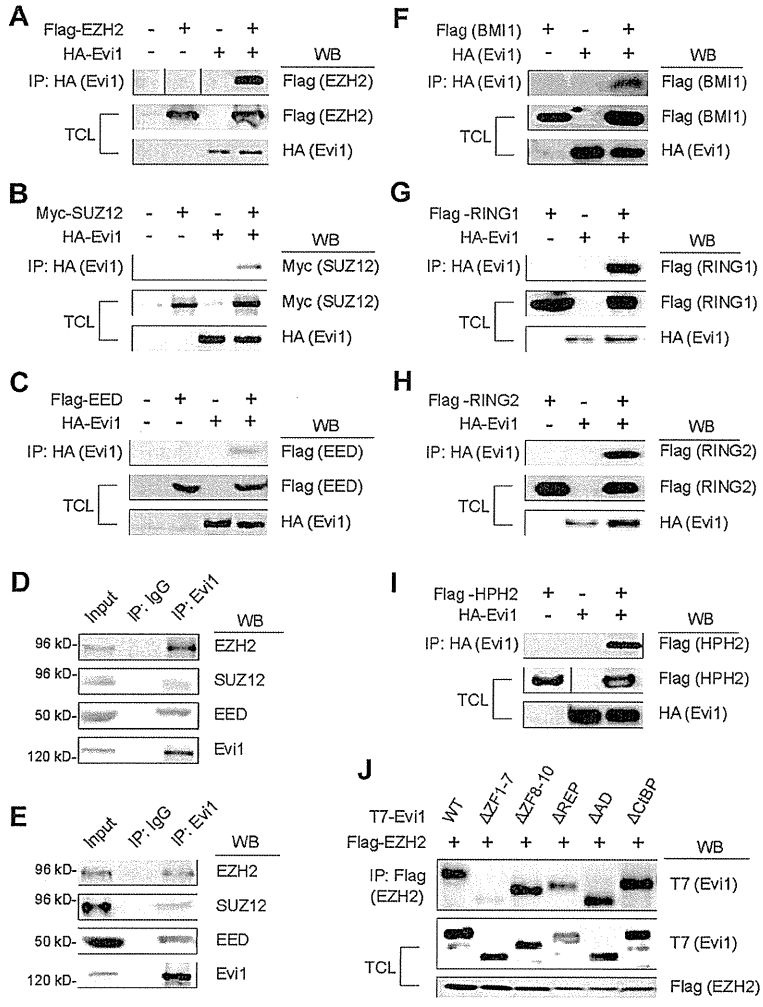

Figure 6. Evi1 interacts with PcG proteins. (A-C) Immunoprecipitation of HA-Evi1 identified EZH2 (A), SUZ12 (B), and EED (C) as interacting proteins in 293T cells. Vertical lines have been inserted to indicate a repositioned gel lane in panel A. (D) Interaction between Evi1 and endogenous PRC2/3/4 proteins in Evi1-induced murine leukemic cells. (E) Interactions between endogenous Evi1 and PRC2/3/4 in human leukemia cells derived from the patient with AML patient. (F-I) Immunoprecipitation of HA-Evi1 identified BMI1 (F), RING1 (G), RING2 (H), and HPH2 (I) as interacting proteins in 293T cells. A vertical line has been inserted to indicate a repositioned gel lane in panel I. (J) ZF1-7 domain of Evi1 is responsible for the physical interaction with EZH2. IgG indicates immunoglobulin G; WB, Western blotting.

basis or drug sensitivity of human leukemia with elevated Evi1 expression. Previous studies reported that Evi1 overexpression induced myelodysplastic syndrome in murine BM transplantation assays,[14,36] and most of the mice died of severe anemia. In contrast, all of the mice that received a transplant in our study died of AML. This discrepancy may derive from the high efficiency of gene transfer into BM cells in our experiments (Figure 1A) or from the difference in virus vectors used in these assays, although other possibilities may exist. Furthermore, the relatively long latency of leukemia development in this mouse model suggests that other genetic events are required for the onset of full-blown leukemia.

We previously showed that Evi1 is essential for proliferation of HSCs and myeloid leukemia cells. It was also shown that inactivation of PTEN in HSCs causes their short-term expansion, but long-term decline, primarily because of an enhanced level of HSC activation.[27,28] Moreover, we observed that PTEN expression was higher in Evi1-deficient HSCs than in wild-type HSCs. Therefore, it is tempting to speculate that the modest down-regulation of PTEN by Evi1 results in HSC expansion without inducing its exhaustion. In addition, given that PTEN is also known as a key regulator in leukemic stem cells, activation of Evi1 may contribute to leukemic stem cell generation through PTEN down-regulation. Thus, the role of Evi1-PTEN pathway in normal and malignant stem cells should be clarified in the future.

Acknowledgments

We thank D. Allis, D. Reinberg, Y. Shi, R. Shiekhattar, P. Freemont, M. Bollen, A. Eynde, H. Wang, D. Wotton, P. Farnham, M. Tomasson, K. Yamamoto, T. Kim, D. Rotin, X. Wang, T. Nakamura, T. Inaba, I. Kitabayashi, F. Fuks, and W. Cavenee for providing essential plasmids; S. Ogawa for technical supports of the microarray experiment; Y. Shimamura for expert technical assistance; Kyowa Hakko Kirin Co Ltd for cytokines.

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology (KAKENHI 19679004).

Authorship

Contribution: A.Y., S.G., and M.K. designed the study; A.Y. performed most of the experiments and wrote the paper with S.G. and M.K.; A.Y. and N.W.-O. performed animal studies; A.Y., Y.N., and M.N. performed microarray analyses; S.G., Y.Y., E.N., S.A., T.S., and M.S. participated in plasmid/protein preparation; M.N. and Y.I. provided technical advice and support; T.K. supervised the

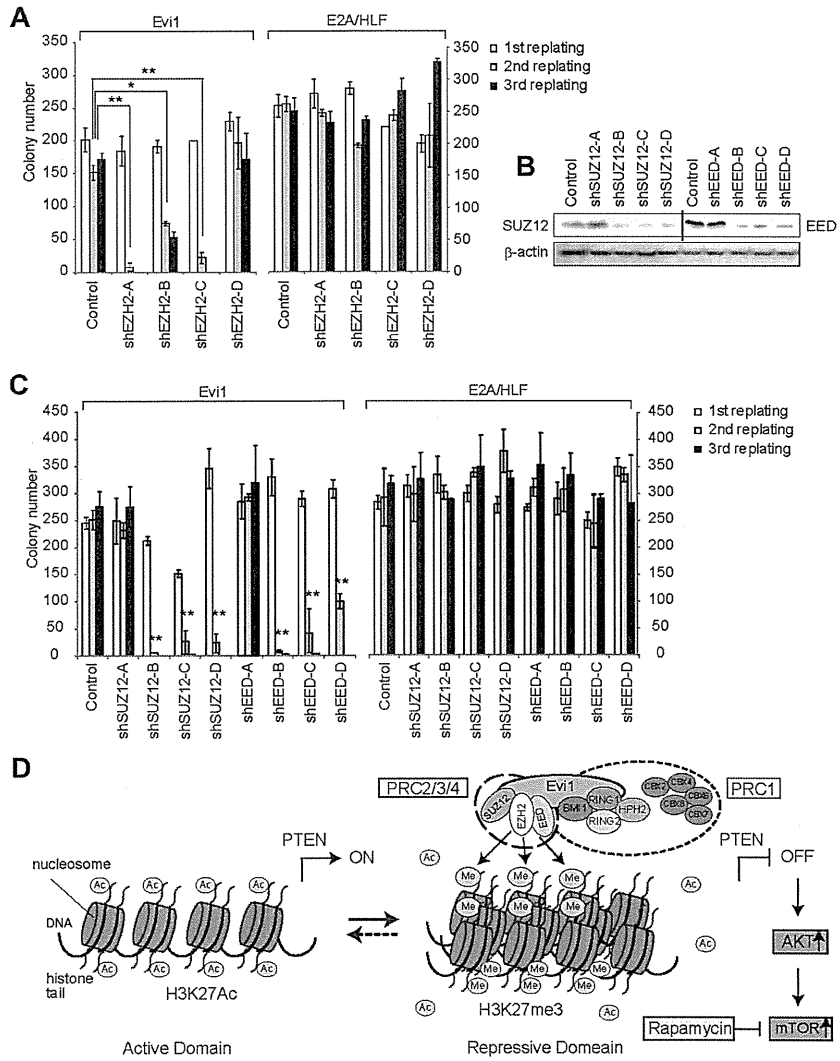

Figure 7. PcG proteins are required for the serial replating capacity of Evi1-transduced BM cells. (A,C) Serial replating capacity of Evi1-expressing cells was markedly reduced with EZH2 (A), SUZ12, or EED (C) shRNAs (n = 8 from 4 independent experiments. Error bars indicate SD; $*P < .01$ and $**P < .001$; *P* values were calculated by comparisons with the colony numbers of control cells at the second replating. (B) RNAi of SUZ12 and EED. (D) A scheme showing that Evi1 recruits polycomb complexes to the PTEN promoter and represses PTEN transcription through H3K27me3-mediated chromatin remodeling, which, in turn, activates downstream AKT/mTOR pathway.

animal studies and assisted with manuscript preparation; and M.K. supervised all of the experiments and data interpretation.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Evi-1 is a transcriptional target of mixed-lineage leukemia oncoproteins in hematopoietic stem cells

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Ecotropic viral integration site-1 (Evi-1) is a nuclear transcription factor that plays an essential role in the regulation of hematopoietic stem cells. Aberrant expression of Evi-1 has been reported in up to 10% of patients with acute myeloid leukemia and is a diagnostic marker that predicts a poor outcome. Although chromosomal rearrangement involving the Evi-1 gene is one of the major causes of Evi-1 activation, overexpression of Evi-1 is detected in a subgroup of acute myeloid leukemia patients without any chromosomal abnormalities, which indicates the presence of other mechanisms for Evi-1 activation. In this study, we found that Evi-1 is frequently up-regulated in bone marrow cells transformed by the mixed-lineage leukemia (MLL) chimeric genes *MLL-ENL* or *MLL-AF9*. Analysis of the Evi-1 gene promoter region revealed that MLL-ENL activates transcription of Evi-1. MLL-ENL–mediated up-regulation of Evi-1 occurs exclusively in the undifferentiated hematopoietic population, in which Evi-1 particularly contributes to the propagation of MLL-ENL–immortalized cells. Furthermore, gene-expression analysis of human acute myeloid leukemia cases demonstrated the stem cell–like gene-expression signature of MLL-rearranged leukemia with high levels of Evi-1. Our findings indicate that Evi-1 is one of the targets of MLL oncoproteins and is selectively activated in hematopoietic stem cell–derived MLL leukemic cells. (*Blood*. 2011;117(23):6304-6314)

Introduction

The ecotropic viral integration site-1 (Evi-1) is a nuclear transcription factor that plays an essential role in the proliferation and maintenance of hematopoietic stem cells (HSCs).[1-3] There are 2 major alternative forms generated from the Evi-1 gene, *Evi-1a* and *Mds1-Evi-1* (also called *Evi-1c*). Mds1-Evi-1 is a fusion variant of Evi-1 generated through intergenic splicing with *Mds1*,[4] a gene located approximately 140 and 500 kb upstream of *Evi-1* in the human and mouse genome, respectively. In contrast to Evi-1a, Mds1-Evi-1 possesses the PRDI-BF1-RIZ1 homologous (PR) domain in the N-terminus, which regulates oligomerization of the Evi-1 proteins.[5] Both Evi-1a and Mds1-Evi-1 are normally coexpressed in several developing and adult tissues,[6] and differences in the normal function between these proteins remain to be elucidated. Like all other PR domain proteins, Evi-1 contains several zinc finger motifs. They are grouped into N-terminal 7 and C-terminal 3 clusters, which are called the first and second zinc finger domain, respectively.[7,8] Between these 2 zinc finger domains lie the C-terminal binding protein (CtBP) domain and the repression domain. The first zinc finger, the repression, and the CtBP-binding domains exhibit a growth-promoting effect by blocking transforming growth factor-β signaling.[9] The first zinc finger domain also exhibits an antiapoptotic effect by repressing c-Jun N-terminal kinase signaling.[10] The second zinc finger domain stimulates proliferation by increasing activator protein-1 activity.[11] Thus, Evi-1 possesses diverse functions as an oncoprotein.

Aberrant expression of EVI-1 frequently has been found in myeloid leukemia and in several solid tumors and is associated with poor prognosis of patients with leukemia.[12-15] Rearrangements of chromosome 3q26, which contains the EVI-1 gene, lead to overexpression of EVI-1 and are implicated in the development or progression of high-risk acute myeloid leukemia (AML).[16] Importantly, EVI-1 is also highly expressed in a subgroup of AML patients without 3q26 rearrangements,[12] which indicates the presence of other mechanisms of EVI-1 activation. Recently, several clinical studies revealed a positive correlation between EVI-1 (both EVI-1a and MDS1-EVI-1) overexpression and rearrangements of the mixed-lineage leukemia (MLL) gene located on chromosome 11q23.[14,15] Furthermore, we have previously shown that Evi-1 deletion in cells transformed by MLL-ENL, a chimeric gene generated in t(11;19) leukemia, caused a distinct reduction of their proliferative activity.[3] These results raise the possibility of functional interaction between Evi-1 and MLL oncoproteins.

The MLL gene encodes a DNA-binding protein that involves the SET [su(var)3-9, enhancer of zeste, and trithorax] domain with histone H3 lysine 4 methyltransferase activity, which regulates gene expression, including multiple Hox genes.[17,18] Chromosome translocations involving the MLL gene are associated with aggressive forms of acute leukemia.[19] Generation of MLL fusion proteins in leukemia deletes the SET domain that mediates histone H3 lysine 4 methylation and fuses the amino portion of MLL in frame with up to 50 different fusion partners, including ENL, AF9, and AF4.[19] It has been shown that several Hox genes are consistently expressed at high levels in MLL-rearranged leukemias, which suggests that MLL oncoproteins inappropriately maintain their expression.[20] Hox proteins form hetero-oligomers with TALE (3–amino-acid loop extension) homeobox proteins of the Pbx and

Submitted July 21, 2009; accepted December 12, 2010. Prepublished online as *Blood* First Edition paper, December 29, 2010; DOI 10.1182/blood-2009-07-234310.

The online version of this article contains a data supplement.

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Meis families, and Meis1 is also highly expressed in MLL-rearranged leukemias.[21,22] A large body of evidence suggests that Hox/Meis genes are crucial targets of MLL oncoproteins in almost all cases of MLL-rearranged leukemias.[22,23] However, MLL-rearranged leukemias are biologically and clinically diverse, and additional factors that underlie these differences have been characterized incompletely.

In the present study, we found that Evi-1 is frequently up-regulated by MLL-ENL or MLL-AF9 in a retroviral transduction assay. The reporter assay and chromatin immunoprecipitation analysis (ChIP) revealed that MLL-ENL binds to and activates the promoter of Evi-1. A retroviral transduction assay with defined populations of bone marrow (BM) progenitor cells revealed that MLL-ENL–mediated Evi-1 up-regulation occurs exclusively in HSCs and not in committed myeloid progenitor cells. These results suggest that up-regulation and maintenance of Evi-1 expression are features of MLL oncoproteins that work specifically in undifferentiated HSCs or progenitor cells.

Methods

Plasmid construction

The plasmids pMSCV-neo-Flag-MLL-ENL, pMSCV-neo-MLL-AF9, pMSCV-neo-AML1-ETO, pMXs-neo-E2A-HLF, and pMYs-HoxA9-ires-Meis1 have been described previously.[3,24-26] The construction procedure is described in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Mice

C57BL/6 mice were purchased from Sankyo Laboratory Service. For the Evi-1 deletion experiments, BM progenitor cells were harvested from wild-type (Evi-1^{+}), loxP-flanked (Evi-1^{f}), and Evi-1–deleted mutant mice (Evi-$1^{+/-}$ and Evi-$1^{f/-}$).[3] Mice were kept at the Center for Disease Biology and Integrative Medicine, University of Tokyo, according to institutional guidelines, and all animal experiments were approved by the University of Tokyo Institutional Animal Care and Use Committee.

Retrovirus transduction

To obtain retrovirus supernatants, Plat-E packaging cells[27] were transfected with retrovirus vectors with FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Viral supernatants were collected after 48 hours of culture and used immediately for infection. To produce green fluorescent protein (GFP)–expressing or Cre-GFP–expressing retrovirus, we used ψMP34 packaging cells (Takara) stably transduced with pGCDN-sam-eGFP or pGCDNsam-eGFP-iCre. Methods to isolate HSCs/progenitor cells from mice are described in supplemental Methods.[28]

Myeloid progenitor transformation assay

The myeloid progenitor transformation assay was performed as described previously[26] with minor modifications. In brief, retrovirus-infected cells were cultured in MethoCult M3434 (StemCell Technologies) and 1 mg/mL G418 at a density of 1×10^5 cells per 35-mm dish. Colonies were counted weekly, and cells were cultured again at 1×10^4 per plate in M3434 without G418. Colony count scoring and replating were repeated every 7 days. For the Evi-1 deletion experiments, BM progenitor cells from Evi-$1^{+/-}$ or Evi-$1^{f/-}$ mice were used.

In vivo leukemogenesis assay

BM mononuclear cells (BM MNCs) harvested from 5-fluorouracil–injected mice were transduced with MLL-ENL or cMyc/bcl2[29] under conditions identical to those for the myeloid progenitor transformation assay. Retrovirally transduced BM progenitor cells (1×10^6) were injected into sublethally irradiated (6.5 Gy) recipients. When transplanted mice became moribund, they were euthanized and their BM MNCs isolated.

Luciferase reporter assay

For analysis of luciferase activities, Jurkat cells were seeded in 12-well culture plates at a density of 0.5×10^5 per well. The cells were transfected with 100 ng of pGL4-Luc or an equimolar amount of each reporter construct, together with 100 ng of pME18S or an equimolar amount of each expression plasmid and 5 ng of PSS-LacZ with FuGENE 6. After 48 hours of culture, cells were harvested and luciferase activities were measured in a Lumat LB9507 luminometer (Berthold Technologies) with a PicaGene luminescence kit (Toyo Ink). Each luciferase activity measurement was normalized to that of β-galactosidase, which was measured with Galacton-Plus (Roche). Data are expressed as mean ± SD from 2 or more separate experiments.

Chromatin immunoprecipitation

ChIP analysis was performed as described previously[30] with minor modifications. The procedures are described in supplemental Methods.

Bioinformatics analyses

The gene-expression pattern in MLL-rearranged leukemia cells was assessed with use of the data of 285 individuals with AML published by Valk et al[14] from the Gene Expression Omnibus (GSE1159). We used 13 cases with MLL rearrangement (after excluding 3 cases with MLL partial tandem duplication) and divided them into 2 groups according to the level of EVI-1 expression: 5 EVI-1–high (GSM20760, 20794, 20838, 20959, and 20966) and 8 EVI-1–low (GSM20757, 20844, 20879, 20891, 20934, 20936, 20938, and 20961) cases. Gene-set enrichment analyses (GSEAs) were performed with GSEA version 2.0 software available from the Broad Institute (http://www.broad.mit.edu/gsea)[31] with a Signal2Noise metric for ranking genes and 1000 data permutations. Functional 1892 gene sets (C2) or 8 selected gene sets that represented HSC and progenitor cell clusters[32] were evaluated (supplemental Methods).

In addition, gene-expression data of murine c-Kit^{+}, Sca-1^{+}, Lin^{-} (KSL) cells and granulocyte-macrophage progenitor cells (GMPs) were obtained from the Gene Expression Omnibus (GSE3725).[33] Dataset comparisons of 13 cases of EVI-1–high or –low leukemias were performed with dChip software (http://www.hsph.harvard.edu/~cli/complab/dchip/).[34] The expression value was calculated by use of a perfect match/mismatch model after transformation into a log2 scale. Differently regulated probe sets in EVI-1–high and –low leukemias were determined by fold change > 1.2, $P < .05$, and 90% lower confidence-bound criteria. In this way, 120 probes enriched in EVI-1–high leukemias and 192 probes enriched in EVI-1–low leukemias were extracted to make gene sets that represented EVI-1–high and –low MLL-rearranged leukemia, respectively (supplemental Table 2). GSEAs were performed with these gene sets and the gene-expression data of murine KSL cells and GMPs.

Statistics

Data were analyzed by Student t test. P values $< .05$ were considered significant.

Results

Evi-1 is up-regulated in myeloid progenitor cells immortalized by MLL oncoproteins

We first assessed Evi-1 expression in myeloid progenitor cells immortalized by various oncogenes. These oncogenes included 2 MLL chimeric genes (MLL-ENL and MLL-AF9), E2A-HLF, and the coexpression of HoxA9 and Meis1. MLL-ENL and MLL-AF9 are major forms of MLL oncoproteins generated in t(11;19) and

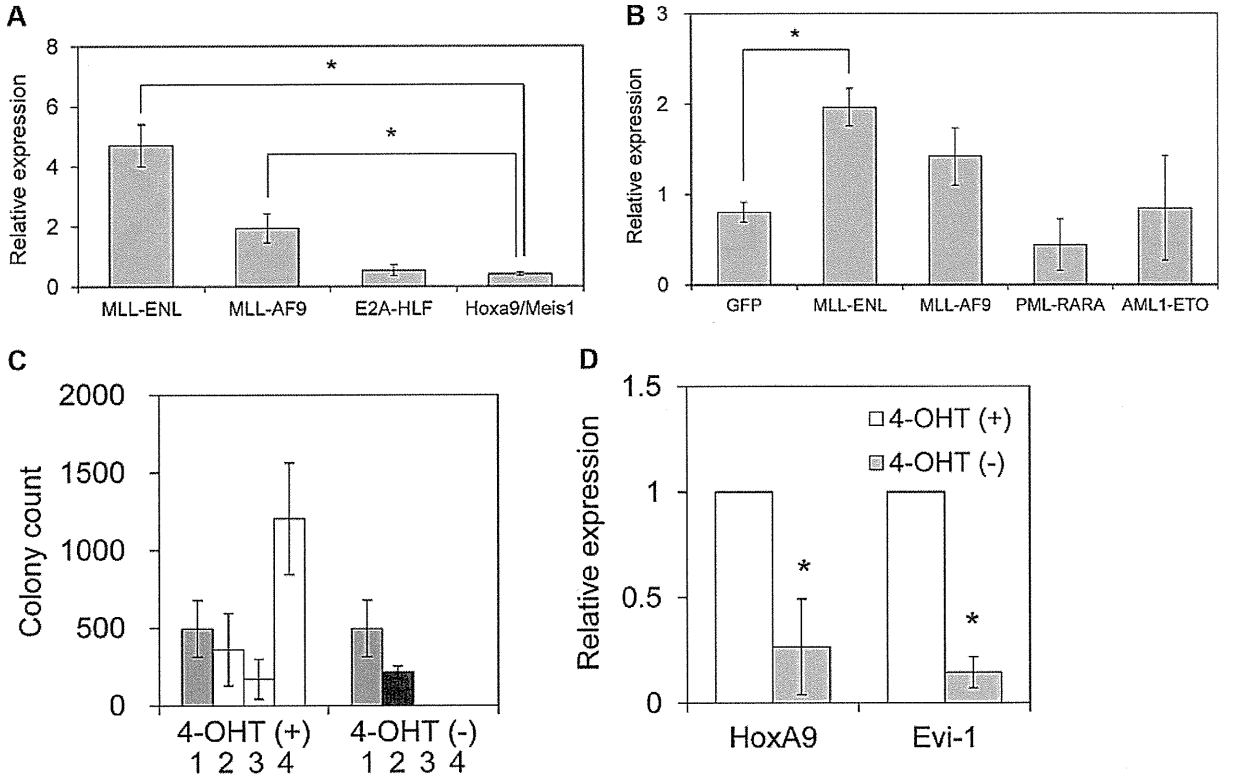

Figure 1. Evi-1 is up-regulated in myeloid progenitor cells immortalized by MLL oncoproteins. (A) Murine c-Kit$^+$ BM progenitor cells were retrovirally transduced with pMXs-neo-MLL-ENL, pMXs-neo-MLL-AF9, pMXs-neo-E2A-HLF, or pMYs-HoxA9-ires-Meis1. The expression level of Evi-1 in immortalized cells from the third to fourth round of serial replating in semisolid medium was quantified relative to BM MNCs with real-time polymerase chain reaction. Data are shown as mean ± SD. *$P < .05$. (B) Murine c-Kit$^+$ BM progenitor cells were retrovirally transduced with leukemia oncogenes. Four types of myeloid leukemia genes cloned into MIG were retrovirally transduced into c-Kit$^+$ BM progenitor cells. Forty-eight hours after initiation of retroviral transduction, GFP-positive cells were isolated and the expression level of Evi-1 was quantified relative to BM MNCs. Data are shown as mean ± SD. *$P < .05$. (C) Immortalization of c-Kit$^+$ BM progenitor cells by MLL-ENL-ER is dependent on the presence of 4-OHT. Graph indicates the number of colonies, with SD, generated from 10^4 pMXs-neo-MLL-ENL-ER–transduced BM cells in the presence (□) or absence (■) of 1 μM 4-OHT at each round after retroviral transduction. The number of G418-resistant colonies obtained by transduction of MLL-ENL-ER into 10^4 BM cells in the presence of 4-OHT is shown in the first round (▨). (D) Expression level of HoxA9 or Evi-1 in MLL-ENL-ER–transformed cells cultured with or without 1 μM 4-OHT for 72 hours. The averages of the relative expression ratio of 4-OHT$^-$ cells (■) to 4-OHT$^+$ cells (□) are shown with SD. *$P < .05$.

t(9;11) leukemias, respectively, that contain nuclear proteins as a fusion partner. E2A-HLF is a chimeric gene generated in t(17;19) leukemia, and it transforms myeloid progenitor cells through Hox-independent mechanisms in mice.[35] HoxA9 and Meis1 are crucial downstream targets of MLL oncoproteins, and coexpression of HoxA9 and Meis1 is sufficient for myeloid transformation.[36] Primary murine hematopoietic progenitor cells (c-Kit$^+$ cells) transduced with these oncogenes, but not those transduced with the empty vector, formed colonies in methylcellulose medium that could be replated through at least 3 rounds of culture (data not shown). After establishment of sustained clonogenic activity after more than 3 rounds of replating, the cells were harvested, and the expression level of Evi-1 was assessed by real-time quantitative polymerase chain reaction analysis. As shown in Figure 1A, Evi-1 was highly expressed in myeloid progenitor cells transformed by MLL-ENL or MLL-AF9 oncoproteins compared with those transformed by HoxA9 and Meis1, which are critical transcriptional targets of MLL oncoproteins. Therefore, HoxA9 and Meis1 appeared unable to complement the transcriptional effect of MLL oncoproteins on Evi-1. On the other hand, Evi-1 was not activated in E2A-HLF–immortalized cells.

Next, we assessed immediate changes in the expression level of Evi-1 in hematopoietic cells induced by transduction of myeloid leukemia genes. We used 2 chimeric genes frequently found in AML: AML1-ETO and PML-RARA. The former is generated in t(8;21) leukemia, whereas the latter is generated in t(15;17) leukemia. We retrovirally transduced these myeloid leukemia genes into BM myeloid progenitor cells. Transduced cells were isolated 48 hours later, and the expression level of Evi-1 was assessed. As shown in Figure 1B, Evi-1 expression was higher in MLL-ENL–transduced BM cells than in GFP-transduced cells, whereas it was not enhanced in either PML-RARA– or AML1-ETO–transduced cells, which are found in the most common forms of myeloid leukemia. Evi-1 was also not significantly up-regulated in MLL-AF9–transduced BM cells.

To determine the dependency of Evi-1 activation on MLL-ENL, we constructed MLL-ENL fused to the estrogen receptor (MLL-ENL-ER), which rendered the transcriptional and transforming properties of MLL-ENL strictly dependent on the presence of 4-hydroxy-tamoxifen (4-OHT). Consistent with a previous report,[23] MLL-ENL-ER–transduced hematopoietic progenitor cells required 4-OHT for myeloid transformation (Figure 1C). Using this system, we quantified the expression of Evi-1 and HoxA9 in MLL-ENL-ER–immortalized cells. By 72 hours after 4-OHT withdrawal, the expression of Evi-1 and HoxA9 was reduced significantly compared with that seen in 4-OHT–positive cells. Thus, inactivation of MLL-ENL results in down-regulation of Evi-1 (Figure 1D), which again suggests a potential relationship between a distinct expression of Evi-1 and MLL-ENL.

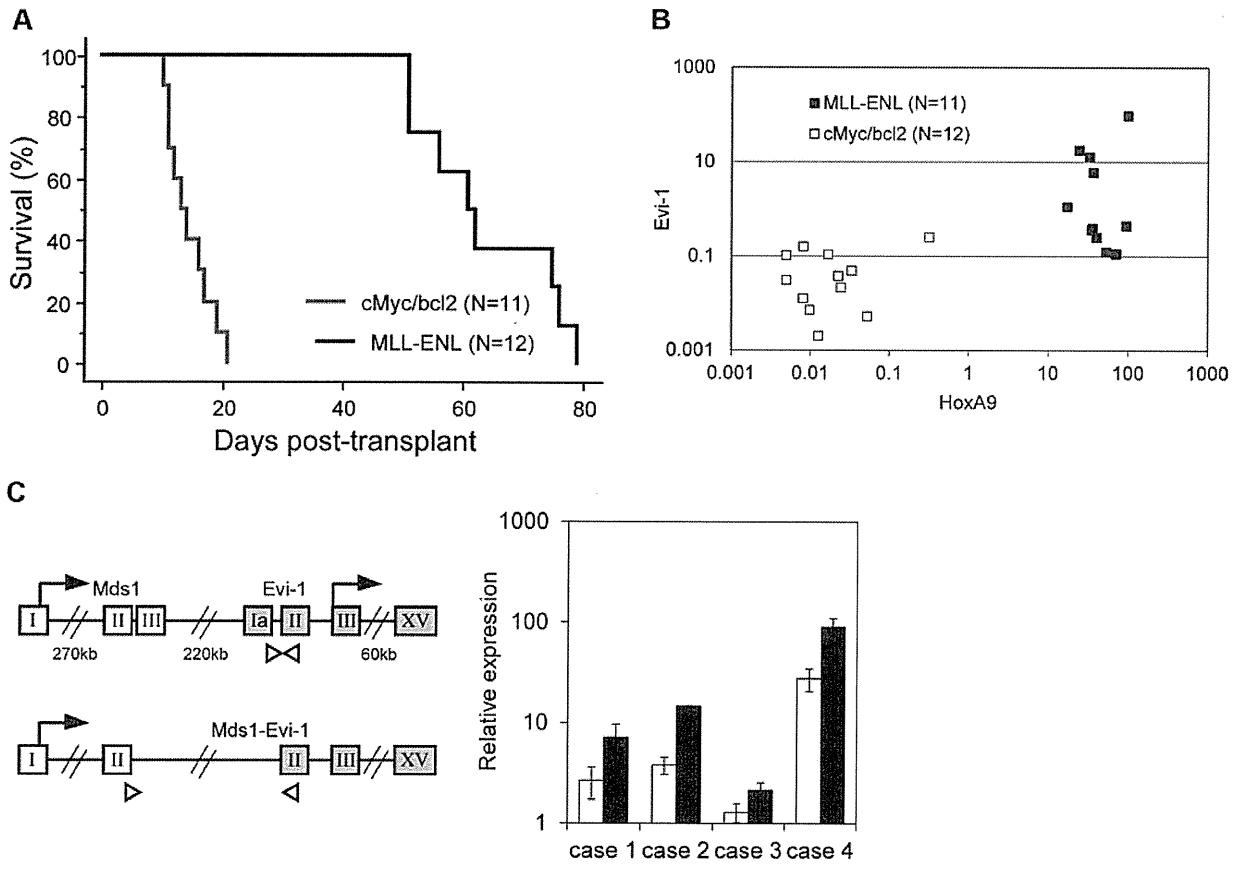

Figure 2. Evi-1 is frequently up-regulated in leukemic cells transformed by MLL fusion protein in vivo. (A) Survival curves of sublethally irradiated recipients transplanted with BM cells transduced with either MSCV-neo-MLL-ENL (n = 11; blue) or MSCV-cMyc-ires-bcl2 (n = 12; red) are shown. (B) Expression levels of Evi-1 and HoxA9 in leukemic cells transformed by MLL fusion genes (MLL-ENL; n = 11; ■) or cMyc/bcl2 (n = 12; □) are indicated. Expression levels of Evi-1 and HoxA9 relative to BM MNCs are shown as squares. (C left) Gene structures of Evi-1a and Mds1-Evi-1 and positions of primer sets for quantitative real-time polymerase chain reaction are shown. The exons, start codons, and primers are depicted in boxes, with arrows, and with white triangles, respectively. Sequences of primers are presented in the supplemental Methods. (Right) Expression levels of Evi-1a (□) and Mds1-Evi-1 (▨) in leukemic cells from 4 mice with high Evi-1 expression relative to BM MNCs are shown with SD.

Expression of Evi-1 in MLL fusion-transformed leukemic cells in vivo

We next assessed Evi-1 expression in leukemic cells transformed by MLL-ENL using a mouse leukemia model. As a control, we used cMyc/bcl2-induced biphenotypic leukemia, the BM infiltration of which consists of a large number of myeloblasts and a small number of lymphoblasts. We harvested BM MNCs from mice treated with 5-fluorouracil. These cells were transduced with MLL-ENL or cMyc/bcl2 and then intravenously injected into sublethally irradiated recipient mice. Mice transplanted with MLL–ENL– or cMyc/bcl2–transduced cells developed leukemia within 85 or 26 days, respectively, which is consistent with previous reports (Figure 2A).[29,37] Leukemic cells were isolated from BM of moribund mice, and the expression level of Evi-1 was determined along with that of HoxA9, a well-known target of MLL-ENL. As shown in Figure 2B, Evi-1 was distinctly up-regulated in MLL-transformed leukemic cells of 4 of the 11 mice but was never activated in cMyc/bcl2–transformed cells ($P = .037$). The expression level of Evi-1 varied considerably among individuals, whereas that of HoxA9 was similar (Figure 2B). These results suggest that the regulation of Evi-1 is independent of HoxA9. Because the Evi-1 gene gives rise to 2 major alternative forms, Evi-1a and Mds1-Evi-1 (Evi-1c), we then assessed the expression of those Evi-1 isoforms using specific primers to detect respective forms. Interestingly, in all 4 individuals with high Evi-1 expression, the expression of both isoforms, Evi-1a and Mds1-Evi-1, was up-regulated (Figure 2C).

MLL oncoproteins specifically up-regulate Evi-1 through 5′ promoter regions

To determine whether MLL-ENL regulates the transcription of Evi-1, we performed a luciferase reporter assay. Because the genomic region 5.7 kb upstream of the transcription start site (TSS) of Evi-1a is highly conserved among species (supplemental Figure 1), we divided the region into 5 fragments and inserted them upstream of luciferase cDNA in the pGL4-Basic vector (Figure 3A). Each reporter plasmid was transfected into Jurkat cells along with the MLL-ENL expression plasmid. MLL-ENL exhibited the greatest increase in luciferase activity with pGL4-E2265 (Figure 3A). These data suggest that MLL-ENL up-regulates Evi-1a through the region that lies between −2.3 and −1.3 kb of the TSS. We next cloned 3 fragments within the genomic region around the Mds1-Evi-1 TSS, which is also evolutionally conserved (Figure 3B; supplemental Figure 1). The luciferase reporter assay revealed that MLL-ENL activated Mds1-Evi-1 transcription mainly through the region between −0.1 and 0.3 kb of the TSS (Figure 3B).

To further confirm the crucial region for Evi-1a activation by MLL-ENL, we generated a series of pGL4-E2265 deletions and

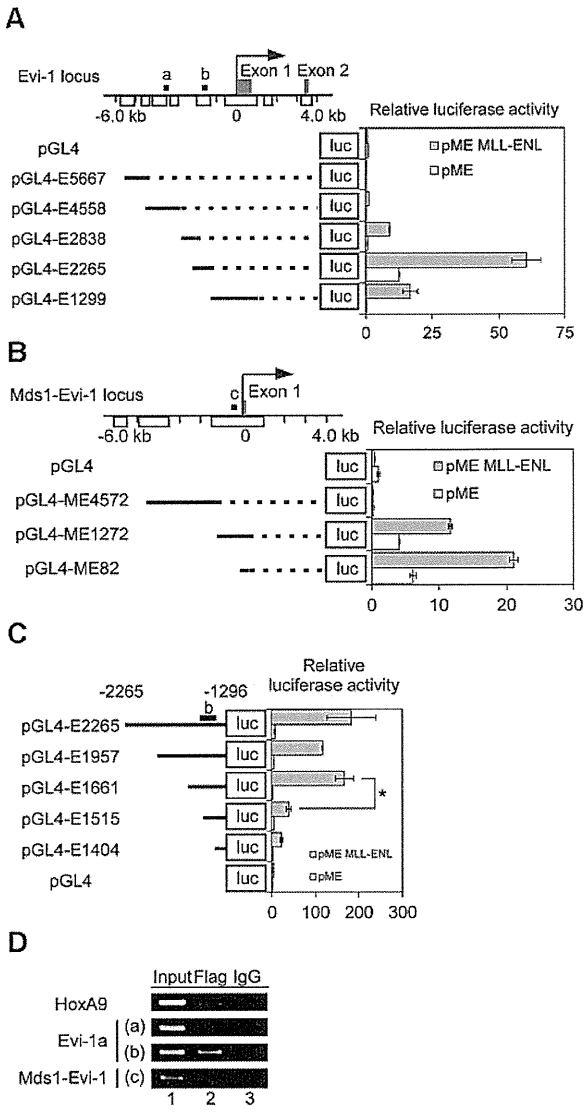

Figure 3. MLL-ENL binds to the promoter regions of both Evi-1a and Mds1-Evi-1. (A left) Five segments of the Evi-1a promoter were inserted upstream of the luciferase cassette of the pGL4-Luc vector to generate luciferase reporter constructs. Arrows, gray boxes, white boxes, solid lines, and dashed lines represent TSS, exons, highly conserved regions between human and mice, DNA sequences cloned into pGL4-Luc, and connection of each DNA segment and luciferase gene, respectively. (Right) Graph shows relative luciferase activity of Evi-1a promoter reporter constructs in Jurkat cell lysates with transiently transfected MLL-ENL (shaded bars) compared with that without MLL-ENL (open bars). Data shown are mean ± SD from 3 independent experiments. (B left) 3 segments of Mds1-Evi-1 promoter were inserted upstream of the luciferase cassette of pGL4-Luc. (Right) Graph shows relative luciferase activity of Mds1/Evi-1 promoter reporter constructs in Jurkat cell lysates with transiently transfected MLL-ENL (shaded bars) compared with that without MLL-ENL (open bars). Data shown are mean ± SD from 3 independent experiments. (C left) Serial deletions of pGL4-E2265 were constructed. The pGL4-E1957 through pGL4-E1404 constructs are named according to the base length between the N-terminal residue of inserted fragments and TSS of Evi-1a on murine genome. The DNA fragment inserted in pGL4-E2265 corresponds to the genomic region that is between 2265 and 1296 bp upstream of the TSS of Evi-1a. (Right) Experiments were performed as described in panel A. Data are representative of 3 independent experiments and shown as mean ± SD. $*P < .05$. (D) Enrichment of MLL-ENL to the promoter of Evi-1a and Mds1-Evi-1 was detected by ChIP. Genomic DNA fragments were immunoprecipitated with anti-Flag antibody (lane 2) or normal mouse IgG (lane 3) from formaldehyde-fixed leukemic cells transduced with Flag-MLL-ENL. DNA fragments containing the indicated promoter regions of Evi-1a or Mds1-Evi-1 were amplified by polymerase chain reaction. The positions of the amplified regions in Evi-1a or Mds1-Evi-1 promoters (labeled a, b, and c) are shown in Figure 3A, B, or C, respectively. For controls, each genomic region was amplified from 1% of purified DNA after formaldehyde fixation and sonication (input, lane 1). Representative data of 4 experiments are shown.

performed a luciferase reporter assay (supplemental Methods). We observed no significant changes of luciferase activity between pGL4-E2265 and E1661 (Figure 3C). In contrast, a remarkable reduction of luciferase activity was observed with the deletion of the N-terminal 146 bases from pGL4-E1661 (Figure 3C). These data suggest that the responsive elements for MLL-ENL are within 1.7 and 1.5 kb upstream of the Evi-1a TSS.

To determine whether MLL-ENL binds to these genomic regions in vivo, we performed ChIP with lysates from MLL-ENL–transformed cells collected from the mice with leukemia. The ChIP assay demonstrated that MLL-ENL bound to the 5′ promoter regions of both Evi-1a and Mds1-Evi-1, which are responsible for activation in the reporter assay, but not to the irrelevant region (Figure 3D).

MLL oncoproteins, but not wild-type MLL, activate the promoter of Evi-1

We then performed the luciferase assay using another MLL-associated gene, MLL-AF9, and several other leukemia-associated genes (PML-RARA, AML1-ETO, E2A-HLF, and the combination of HoxA9 and Meis1). MLL-AF9 exhibited transcriptional activity comparable with that of MLL-ENL on the Evi-1 promoter (Figure 4A). In contrast, PML-RARA, AML1-ETO, E2A-HLF, and HoxA9/Meis1 exhibited minimal or no transcriptional activity on the Evi-1 promoter (Figure 4A), which is in agreement with the results of the expression analysis of Evi-1 in BM cells (Figure 1A-B).

Because wild-type MLL also transcriptionally activates its targets such as Hox genes, we tested its transcriptional activity on the Evi-1 promoter. We observed no significant transcriptional activities of wild-type MLL on the Evi-1 promoter, which suggests that wild-type MLL by itself is not sufficient for activation of Evi-1 (Figure 4A).

Next, we determined the domain contribution of MLL-ENL in the activation of Evi-1 using a series of MLL-ENL mutants (Figure 4B). The CXXC domain of MLL mediates binding to nonmethylated CpG DNA and is essential for myeloid transformation.[38] The AT-hook motifs of MLL are thought to facilitate binding to AT-rich DNA in the minor grove but are dispensable for myeloid transformation.[38] Consistent with its contribution to the transforming activity of MLL-ENL, the CXXC domain was essential for Evi-1 activation (Figure 4C). Unexpectedly, deletion of the AT-hook motifs affected reporter activity, although the AT-hook motif was dispensable for MLL-ENL–mediated myeloid transformation and up-regulation of Hox genes, and the menin-binding motif was not required for Evi-1 activation (Figure 4C).[39]

Evi-1 is up-regulated by MLL-ENL exclusively in HSC-derived transformed cells

Although clinical studies revealed a positive correlation between high Evi-1 expression and MLL rearrangements, there exists a subset of MLL-rearranged leukemia with normal Evi-1 expression levels.[14,15] We also observed that the expression level of Evi-1 in MLL cells varied considerably among the individual mice (Figure 2B). Because Evi-1 is preferentially expressed in HSCs and the expression level decreased on differentiation,[2] we hypothesized that Evi-1 expression in MLL cells depends on their cellular origin.

To test this hypothesis, we transduced MLL-ENL into the defined hematopoietic populations, including KSL cells, which contain HSCs, myeloid-restricted common myeloid progenitor cells (CMPs), and GMPs.[28] Consistent with a previous report, MLL-ENL immortalized committed myeloid progenitor cells (CMPs

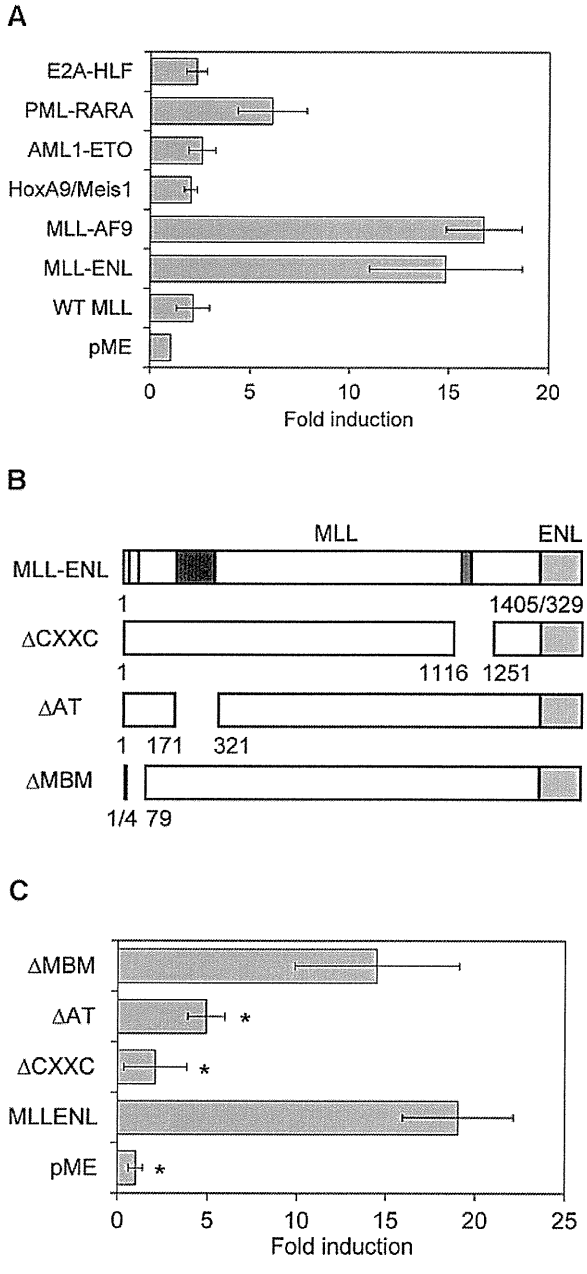

Figure 4. Reporter assays using MLL-ENL deletion mutants and other oncoproteins. (A) Transactivation of pGL4-E2265 induced by MLL-AF9, AML1-ETO, PML-RARA, E2A-HLF, HoxA9/Meis1, or wild-type MLL (WT MLL) is shown. Data are presented as a relative fold increase in mean luciferase activity, with SD, after adjustment for β-galactosidase activity. (B) Schematics represent the composition of MLL-ENL deletion mutants. Numbers denote amino acid positions in wild-type MLL and ENL. Positions of CXXC DNA binding motif (red), AT-hooks DNA binding motif (AT; blue), and menin-binding motif (MBM; yellow) are shown in the schematics of intact MLL-ENL. (C) Transactivation of pGL4-E2265 induced by intact MLL-ENL or its deletion mutants is shown. Data are presented as described in (A). $^*P < .05$ versus MLL-ENL.

and GMPs), as well as KSL cells (data not shown).[40] After 3 rounds of replating in semisolid medium, we compared expression levels of Evi-1 and HoxA9 in MLL-ENL–immortalized cells derived from KSL cells, CMPs, and GMPs. Notably, Evi-1 expression was significantly high in KSL-derived MLL-ENL–immortalized cells compared with CMP- and GMP-derived cells immortalized by MLL-ENL (Figure 5A). In contrast, HoxA9 was similarly upregulated in the 3 populations (Figure 5A).

We next evaluated the effect of HoxA9/Meis1 on the expression of Evi-1 in the 3 hematopoietic populations noted above. HoxA9/Meis1 transformed KSL cells (data not shown), whereas CMPs and GMPs were not transformed in our experiments. Consistent with our results in the myeloid progenitor transformation assay, KSL cells transformed by HoxA9/Meis1 exhibited a low level of expression of Evi-1 (Figure 5A). These data indicate that Evi-1 is not a transcriptional target of HoxA9 or Meis1 even in KSL cells.

To identify whether Evi-1 transcription in KSL cells is activated or maintained by MLL-ENL, we first assessed immediate changes in the Evi-1 expression level induced by MLL-ENL. We retrovirally transduced MLL-ENL into KSL cells and GMPs. Transduced cells were isolated 48 hours later, and the expression level of Evi-1 was assessed. Remarkably, Evi-1 expression in MLL-ENL–transduced KSL cells was significantly higher than that in freshly isolated KSL cells (Figure 5B). On the other hand, MLL-ENL–transduced GMPs showed low expression of Evi-1 compared with freshly isolated GMPs (Figure 5B).

To further examine the transcriptional regulation of Evi-1 in undifferentiated hematopoietic cells, we retrovirally transduced MLL-ENL into BM MNCs from 5-fluorouracil–treated mice in which HSCs were propagated. Thirty-six hours later, transduced cells were isolated from the KSL population. Consistent with the results shown in Figure 5B, MLL-ENL–transduced KSL population cells exhibited significantly higher expression of Evi-1 than freshly isolated KSL cells (Figure 5C).

We also examined the transcriptional regulation of Evi-1 by MLL-AF9, because MLL-AF9 exhibited a transcriptional activity comparable with that of MLL-ENL on the Evi-1 promoter (Figure 4A). Transduction analysis with undifferentiated hematopoietic cells clearly demonstrated that MLL-AF9 activates Evi-1 expression in the same manner as MLL-ENL (Figure 5B-C). Collectively, our results show that Evi-1 transcription is not only maintained but also activated by MLL-ENL or MLL-AF9 exclusively in undifferentiated hematopoietic populations such as KSL cells.

Propagation of MLL-ENL–immortalized HSCs is highly dependent on Evi-1

Previous studies showed that Evi-1 is required for efficient propagation of MLL-ENL–immortalized BM cells.[3] However, it had not been assessed whether the requirement for Evi-1 differs depending on the cellular origin that is immortalized by MLL-ENL. To address this issue, BM progenitor cells from Evi-$1^{+/-}$ and Evi-$1^{f/-}$ mice were sorted into KSL cells, CMPs, and GMPs. Then, they were transduced with MLL-ENL and immortalized via a myeloid transformation assay. First, using Evi-$1^{+/-}$ cells, we revealed that deletion of 1 Evi-1 allele had no significant impact on the clonogenic activity of each hematopoietic population (Figure 5D). Next, using Evi-$1^{f/-}$ cells and Cre-GFP retrovirus, we completely disrupted Evi-1 alleles in MLL-ENL–immortalized cells derived from the defined populations (supplemental Figure 2). Then, GFP- or Cre-GFP–infected cells were sorted and cultured for another round in semisolid medium to compare the effects of Evi-1 deletion on the clonogenic activity among populations. Notably, colony formation of MLL-ENL–immortalized cells derived from KSL cells was most severely attenuated by disruption of Evi-1, compared with cells derived from CMPs or GMPs (Figure 5E). On subsequent replating of Evi-1–deleted cells, however, we observed no significant difference in colony counts among populations (supplemental Figure 2). These results indicate that MLL-ENL–

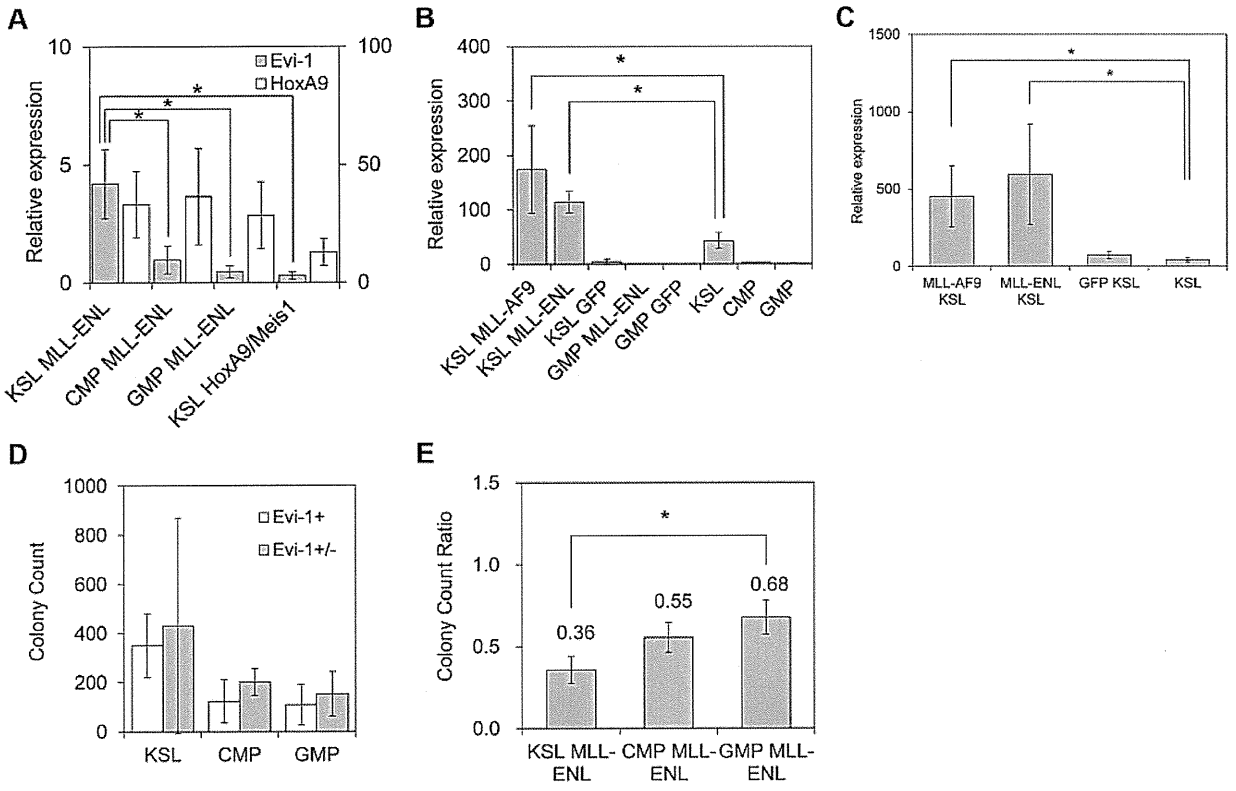

Figure 5. MLL-ENL up-regulates Evi-1 expression exclusively in HSCs. (A) Defined hematopoietic populations were transduced with pMXs-neo-MLL-ENL or pMYs-HoxA9-ires-Meis1 and replated in semisolid medium. The expression level of Evi-1 (shaded bars; scale on the left) and HoxA9 (open bars; scale on the right) in MLL-ENL–transformed cells from each population (KSL MLL-ENL, CMP MLL-ENL, or GMP MLL-ENL lanes) and HoxA9/Meis1-transformed KSL cells (KSL HoxA9/Meis1 lane) was quantified relative to BM MNCs. Data are shown as mean ± SD from 2 independent experiments. $^*P < .05$ vs CMP MLL-ENL, GMP MLL-ENL, or KSL HoxA9/Meis1, respectively. (B) KSL cells and GMPs were transduced with MIG (KSL/GMP GFP lanes), MIG-MLL-ENL (KSL/GMP MLL-ENL lanes), or MIG-MLL-AF9 (KSL MLL-AF9 lane). After 48 hours of transduction, the expression level of Evi-1 in GFP-positive cells was quantified relative to BM MNCs by real-time polymerase chain reaction and was compared with that of freshly isolated KSL cells (KSL lane), CMPs, and GMPs. Data shown are mean ± SD from 3 independent experiments. $^*P < .05$. (C) BM progenitor cells from 5-fluorouracil–treated mice were transduced with MIG (GFP KSL lane), MIG-MLL-ENL (MLL-ENL KSL lane), or MIG-MLL-AF9 (MLL-AF9 KSL lane). After 36 hours of transduction, the expression level of Evi-1 in GFP-positive cells isolated from the KSL population was quantified relative to BM MNCs and compared with that of freshly isolated KSL cells (KSL lane). Data are mean ± SD from 3 independent experiments. $^*P < .05$. (D) BM KSL cells, CMPs, and GMPs were isolated from Evi-1$^+$ (open bars) and Evi-1$^{+/-}$ (shaded bars) mice and transformed by MLL-ENL in the same way as in the myeloid progenitor transformation assay. Bar graph shows mean colony numbers ± SD in the third round of serial replating from 2 independent experiments. $^*P < .05$. (E) BM KSL cells, CMPs, and GMPs were isolated from Evi-1$^{f/-}$ mice. After they were transformed by MLL-ENL as in (D), they were transduced with either pGCDNsam-eGFP or pGCDNsam-eGFP-iCre. GFP-positive cells were isolated, and colony-forming activity after Evi-1 deletion was assessed in the next round of plating. Bar graph shows colony count ratio of iCre-GFP–transduced cells compared with GFP-transduced cells. Data are mean ± SD from 2 independent experiments. $^*P < .05$.

immortalized cells are heterogenous with regard to dependency on Evi-1 for proliferation, even if they are derived from KSL cells. The frequency of Evi-1–dependent cells should be highest in HSC-derived cells and low in progenitor cell–derived cells. Thus, if colony counts decrease immediately after Evi-1 deletion in HSC-derived cells, the residual cells, most of which are no longer dependent on Evi-1, would show almost the same clonogenic activity as progenitor cell–derived cells in the next round.

HSC genes are enriched in EVI-1–high cases of MLL-rearranged leukemia

On the basis of the finding that up-regulation of Evi-1 in MLL fusion-transformed cells is related to their origin, we hypothesized that the gene-expression pattern in human cases of MLL-rearranged leukemia would also reflect their origin. To address this issue, we extracted the gene-expression data of 13 MLL-rearranged AML patients reported by Valk et al from the Gene Expression Omnibus[14] and divided them into 2 groups: 5 EVI-1–high cases and 8 EVI-1–low cases. We then applied GSEA to identify functional gene sets (C2) correlated with EVI-1 expression and found that 2 and 68 gene sets were particularly enriched in the EVI-1–high and EVI-1–low groups, respectively (false-discovery rate < 0.01; gene sets consisting of < 30 genes were excluded; supplemental Table 1). Of note, GSEA revealed a strong correlation of genes up-regulated in EVI-1–high leukemias with the gene set that identifies HSCs (Figure 6Ai). Conversely, the genes down-regulated in EVI-1–high leukemias were strongly correlated with a gene set that is highly expressed in progenitor cells (Figure 6Aii). We also found another gene set that contained long-term HSC-enriched genes was significantly correlated to genes up-regulated in EVI-1–high leukemias (Figure 6B).

In addition, we extracted the expression data of murine normal KSL cells and GMPs and applied GSEA using 2 gene sets that represented EVI-1–high and EVI-1–low MLL-rearranged leukemias, respectively (supplemental Table 2). As expected, GSEA showed a significant correlation of genes enriched in EVI-1–high leukemias with those in KSL cells (Figure 6C), although the reverse correlation between genes representing EVI-1–low leukemias and GMPs was not significant (supplemental Figure 3). These results are consistent with our findings that Evi-1 is up-regulated by

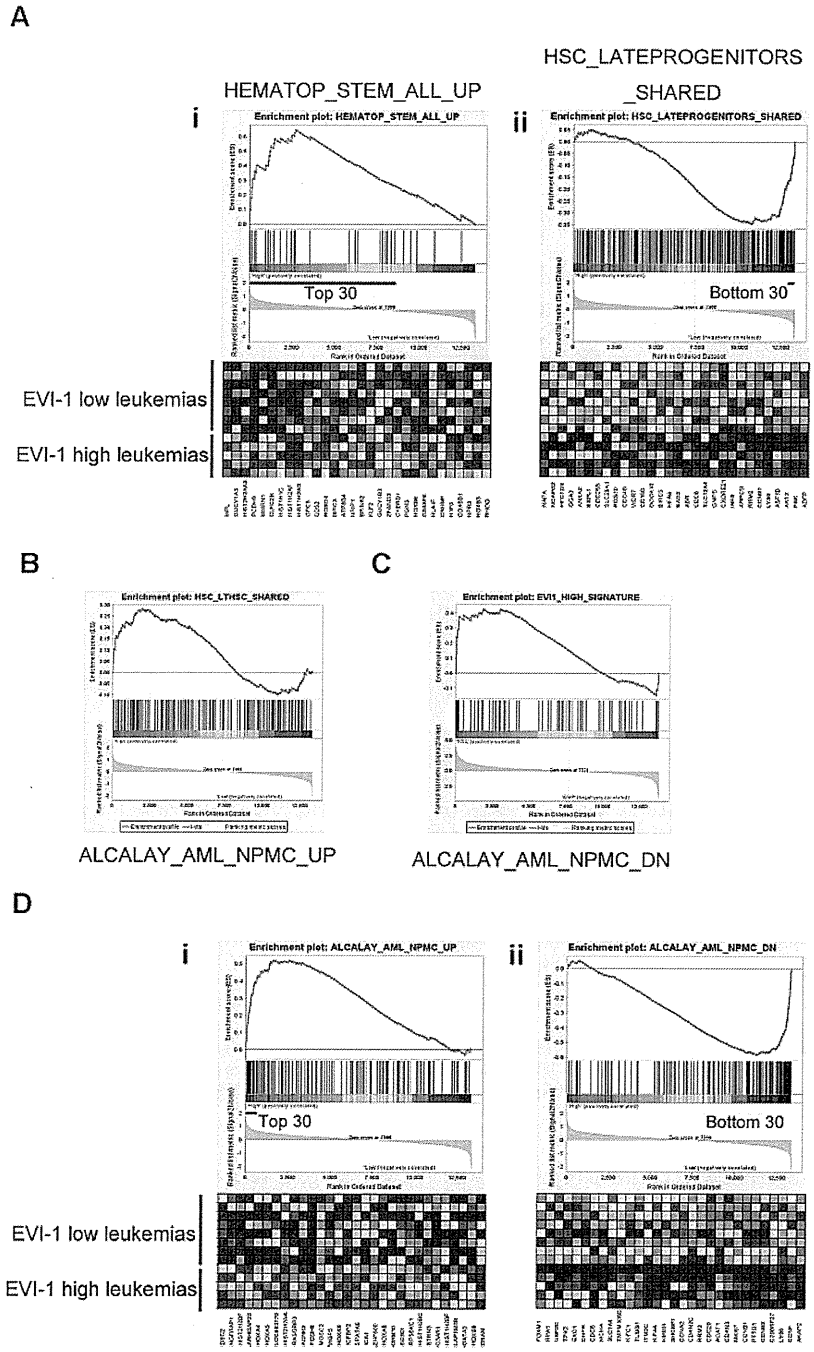

Figure 6. Gene-expression profiles of Evi-1–high leukemias with MLL rearrangement revealed a stem cell–like character. (A) A strong correlation of up-regulated genes in EVI-1–high leukemias with a gene set representing HSCs and of down-regulated genes in EVI-1–high leukemias with a gene set typical of progenitor cells is shown. GSEA of gene expression in human EVI-1–high AMLs with MLL rearrangement (n = 5) was compared with EVI-1–low AMLs with MLL rearrangement (n = 8) using functional gene sets (C2). (Ai,ii) GSEA enrichment plots of the selected gene sets. (Ai) Up-regulated genes in human HSCs.[41] (Aii) Up-regulated genes in hematopoietic late progenitor cells.[32] The corresponding heat maps represent expression of the 30 leading genes of the respective gene sets. Up-regulated and down-regulated genes are shown in red and blue, respectively. (B) GSEA plots show that expression of genes representing long-term HSCs (LTHSC) is enriched in EVI-1–high AMLs with MLL rearrangement compared with EVI-1–low AMLs with MLL rearrangement. Normalized enrichment score = 1.33; false-discovery rate q = .141. (C) GSEA of gene expression in 5 normal KSL samples and 4 normal GMP samples using a gene set representing EVI-1 high leukemia with MLL rearrangement. GSEA plots show that expression of genes representing EVI-1–high leukemia is enriched in KSL cells compared with GMPs. Normalized enrichment score = 1.46; false-discovery rate q = .025. (D) A strong correlation of up-regulated genes in EVI-1–high leukemias with those in cytoplasmic nucleophosmin–positive (NPMc$^+$) leukemias existed. GSEA of gene expression in human EVI-1–high AMLs with MLL rearrangement (n = 5) was compared with EVI-1–low AMLs with MLL rearrangement (n = 8) using functional gene sets (C2). (Di-ii) GSEA enrichment plots of the selected gene sets. (Di) Up-regulated genes in NPMc$^+$ leukemias.[42] (Dii) Down-regulated genes in NPMc$^+$ leukemias.[42]

MLL oncoproteins in HSCs and suggest that EVI-1–high MLL-rearranged leukemias are derived from HSCs. GSEA also demonstrated strong correlation between the up-regulated genes in AMLs with aberrant cytoplasmic localization of nucleophosmin (NPMc$^+$ AMLs) and those in EVI-1–high cases of MLL-rearranged AMLs (Figure 6Di-ii; supplemental Table 1).

Discussion

Despite the established role of Evi-1 in leukemogenesis, the molecular mechanisms for Evi-1 activation in leukemic cells have been poorly understood. Recently, several clinical studies revealed a positive correlation between EVI-1 overexpression and MLL rearrangements in AML patients.[14,15] Furthermore, we have previously shown that Evi-1 deletion in MLL-ENL–immortalized cells caused a distinct reduction of their colony-forming capacity, which suggests a functional interaction between Evi-1 and MLL oncoproteins.[3] In the present study, we demonstrated that MLL oncoproteins activate transcription of the Evi-1 gene in hematopoietic cells. Importantly, this MLL-mediated Evi-1 activation occurs exclusively in HSCs and not in committed myeloid progenitor cells.

Mds1 is located approximately 140 and 500 kb upstream of the first exon of Evi-1 in the human and mouse genome, respectively; therefore, the expression of Evi-1a and Mds1-Evi-1 is regulated by different promoters. Nevertheless, MLL oncoproteins bind to the promoters of both Evi-1a and Mds1-Evi-1 and activate their transcription (Figure 3A-B,D). These findings are consistent with the clinical observations that showed that expression of both EVI-1a and Mds1-EVI-1 is frequently enhanced in MLL-rearranged leukemia.[15] Although some evidence suggests that Evi-1a is oncogenic and Mds1-Evi-1 contributes to tumor suppression,[12,43] several reports showed that the activating retroviral insertions in the Mds1/Evi-1 locus were involved in long-term dominance in hematopoiesis, which suggests a similar function of Mds1/Evi-1 and Evi-1.[44,45] The specific roles of Evi-1a and Mds1-Evi-1 in MLL-rearranged leukemia should be clarified in future studies.

We found a genomic region, 146 bp in length, that is thought to be crucial for Evi-1a activation by MLL-ENL (Figure 3C). The ChIP assay revealed that DNA binding of MLL-ENL was enriched near this genomic region, which also suggests the importance of this region for MLL-ENL to regulate Evi-1 (Figure 3D). To identify the precise genomic DNA sequence to which MLL binds, we performed an electrophoresis mobility shift assay using 3 probes from the genomic region with purified His-tagged protein that contained the MLL-ENL CXXC domain. In this setting, we observed a sequence-specific shifted band using 1 of 3 probes; however, it was not supershifted by the addition of His antibodies (data not shown). These results may be due to some technical difficulties in protein-antibody binding in native conditions. Therefore, it remains to be determined whether this region fragment is sufficient for MLL-ENL to bind to DNA or whether other regions are also involved.

Several studies showed that both HSCs and committed myeloid progenitor cells could be transformed by retroviral transduction of MLL oncoproteins, and they could develop immunophenotypically similar AML.[40] Of note, we found that Evi-1 was activated by MLL-ENL or MLL-AF9 exclusively when it was transduced into KSL cells (Figure 5A-C). Previously, Chen et al[46] reported that HSCs from MLL-AF9 knock-in mice express high levels of Evi-1. In light of that report and the present results, the cellular milieu provided by HSCs appears necessary for Evi-1 up-regulation by MLL oncoproteins. One possibility that accounts for these phenomena is that MLL-ENL can bind to the promoter region of Evi-1 only in HSCs. However, using ChIP assay, we found that MLL-ENL bound to the promoter regions of Evi-1a and Mds1-Evi-1 even in leukemic cells with low Evi-1 expression (data not shown). Therefore, binding to the Evi-1 promoter alone is not sufficient for activation of the transcription of Evi-1. The methylation status of the Evi-1 promoter can affect the expression of Evi-1. To address this issue, we analyzed the methylation status of the Evi-1 promoter in MLL-ENL–transformed cells using bisulfite DNA sequencing. However, the methylation status at CpGs was largely low in the Evi-1 promoter, regardless of Evi-1 expression levels (supplemental Figure 4). On the basis of these findings, it is unlikely that the expression of Evi-1 is shut down in progenitor cells by DNA methylation in the promoter. Another possibility is that undifferentiated HSCs irreversibly lose some key factors that contribute to activation of Evi-1 along with hematopoietic cell differentiation. Significant in this regard is that menin is required for some targets to be activated by MLL oncoproteins.[39] In the present case, however, menin itself was not likely to be a key factor in the MLL-mediated activation of Evi-1, because the menin-binding motif was not required for activation of the Evi-1 promoter in the luciferase assay (Figure 4C).

In the reporter assay used in the present study, Jurkat cells provided a condition sufficient for MLL-ENL to activate the Evi-1 promoter. However, only an exogenous Evi-1 promoter was activated by MLL-ENL in Jurkat cells, given that endogenous Evi-1 expression was not concurrently activated (data not shown). The cellular milieu provided by HSCs ultimately appears necessary for activation of the endogenous Evi-1 promoter.

It has been shown that both wild-type MLL and Evi-1 are crucial for proliferation and maintenance of HSCs.[2,3,47,48] Because wild-type MLL and MLL oncoproteins share some transcriptional targets, such as HoxA9, we assessed the transcriptional activity of MLL on the Evi-1 promoter. The luciferase reporter assays used in the present study showed no significant transcriptional activities of wild-type MLL on the Evi-1 promoter, which suggests that the wild-type MLL by itself is not sufficient for the activation of Evi-1 (Figure 4A). Given that Evi-1 expression decreases along with normal hematopoietic cell differentiation in spite of the preserved expression of MLL, the physiologic expression level of wild-type MLL may not be able to activate the expression of Evi-1 by itself.

GSEA analysis with gene-expression data of AML samples revealed that EVI-1–high MLL-rearranged leukemias exhibit HSC-like signatures, whereas genes involved in more differentiated hematopoietic progenitor cells are enriched in EVI-1–low MLL-rearranged leukemias. Considering that HSCs are more efficient targets for leukemogenic transformation by MLL oncoproteins,[40,46] up-regulated Evi-1 may contribute to the propagation of leukemia stem cells in MLL-rearranged leukemias. In support of this concept is our finding that Evi-1 deletion reduces clonogenic activity most severely in KSL-derived cells (Figure 5E).

In addition to the enrichment of HSC genes in Evi-1–high leukemias, GSEA also revealed that the gene-expression signature of NPMc$^+$ AMLs resembles that of Evi-1–high MLL-rearranged AMLs. Because NPMc$^+$ AMLs display a specific gene-expression profile dominated by an HSC molecular signature,[42] the results probably indicate that HSC genes are enriched in Evi-1–high leukemias. Alternatively, Evi-1 overexpression and cytoplasmic NPM may cooperatively contribute to leukemia development, and this possibility should be investigated in the future.

Given that Evi-1 plays an essential role in the proliferation and maintenance of HSCs in normal hematopoiesis,[3] it is tempting to speculate that activated expression of Evi-1 by MLL oncoproteins results in the propagation of leukemia stem cells that is associated with therapeutic resistance and disease progression. In support of this is a recent report that the adverse effect of EVI-1 positivity on prognosis was clinically observed in AML patients with MLL

rearrangement.[49] We showed that MLL-ENL–transformed cells with up-regulated Evi-1 expression are derived from HSCs (Figure 5A-B) and that their clonogenic potential is highly dependent on Evi-1 (Figure 5E). Collectively, our findings suggest that Evi-1 is an attractive therapeutic target in the treatment of Evi-1–high MLL-rearranged leukemias. Putative key factors collaborating with MLL oncoproteins in undifferentiated hematopoietic cells at the Evi-1 promoter remain unknown. Some clues may be found from the clinical experience that high expression of Evi-1 is frequently observed in leukemias with another MLL rearrangement, MLL-AF6.[14] MLL-AF6, as well as MLL-ENL and MLL-AF9, aberrantly recruits AF4 and ENL family proteins to its transcriptional target promoters to cause sustained target-gene expression.[50] These functions, which are common in major MLL oncoproteins, may be involved in activation of Evi-1. Further investigation would clarify how Evi-1 is activated not only in MLL-rearranged leukemias but also in other leukemias or normal hematopoiesis.

Acknowledgments

We thank T. Kitamura for Plat-E packaging cells, H. Nakauchi for pGCDNsam-eGFP retroviral vector, R. Ono and T. Nosaka for MLL-ENL cDNA, J.L. Hess for MLL-AF9 cDNA, T. Nakamura for HoxA9/Meis1 cDNA, T. Inaba for E2A-HLF cDNA, M.H. Tomasson for cMyc/bcl2 cDNA, I. Kitabayashi for PML-RARA cDNA, Y. Ishii and Y. Shimamura for expert technical assistance, and Kyowa Kirin for cytokines.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and by Health and Labor Sciences Research grants from the Ministry of Health, Labor and Welfare.

Authorship

Contribution: S.A., S.G., and M.K. designed the experiments and the study; S.A., M.N., Y.I., S.G., and M.K. wrote the manuscript; S.A., A.Y., and S.G. performed experiments and collected and analyzed data; and M.S. and M.I. provided important reagents and reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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